MECHANISMS OF ACTION OF POLYSPHOSPHAZENE-BASED ADJUVANTS IN PORCINE MONOCYTES

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Kaitlin Zelda Szombathy

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ABSTRACT

Adjuvants are compounds that enhance immune responses to antigens present in a vaccine. They are particularly important in subunit vaccines; without adjuvants, these vaccines are often poorly immunogenic. A novel adjuvant platform developed at VIDO-InterVac is comprised of CpG-ODN or poly I:C, innate defense regulator peptides, and a new class of adjuvant called polyphosphazene. The polyphosphazenes have demonstrated a great potential as a safe and effective adjuvant. In particular, the polysphosphazenes poly[di(carboxylatophenoxy)-phosphazene] PCPP and poly[di(sodium carboxylatoethylpehnoxy)-phosphazene](PCEP) have been used in numerous animal studies where they not only have been shown to enhance the quality and quantity of the adaptive immune response, but also were shown to induce parenteral and mucosal immune responses with many different antigens, demonstrating their versatility. However the mechanisms by which the polyphosphazenes stimulate the innate immune response are only partially understood.

Antigen presenting cells (APCs) are capable of facilitating the uptake of antigen and directing the immune response. Based upon the proposed mechanism of action of another adjuvant, we chose to investigate whether porcine monocytes could be induced to secrete pro-inflammatory cytokines IL-1β and IL-18 in response to stimulation with polyphosphazenes PCEP and PCPP. The release of these cytokines is thought to be mediated by the Nod-Like Receptors (NLRs), which are cytosolic pattern recognition receptors expressed in APCs. It is suggested that these receptors act in conjunction with TLR transcription pathways to control caspase-1 and release associated pro-inflammatory cytokines IL-1β and IL-18 (Kawai and Akira, 2011).

We first investigated the relative gene expression of three Nod-like receptor genes: *nod1, nod2* and *nlrp3* in various populations of porcine peripheral blood mononuclear cells (PBMCs) and found that monocytes, dendritic cells and B cells express increased relative levels of these receptors as compared to T cells. Subsequently, we evaluated the relative NLR expression in several porcine mucosal and lymphoid tissues and observed genes to be most significantly expressed in nasal mucosa, bronchial mucosa, and lung while limited in tissues associated with Peyer's patches, jejunal wall. Both the mesenteric lymph node and bronchial lymph node exhibited similar patterns and levels of expression of *nod1, nod2* and *nlrp3.*

To characterize the activation of NOD1, NOD2 and NLRP3 receptors in response to stimulation with polyphosphazenes, porcine monocytes were stimulated with PCEP or PCPP in both the presence and absence of a second signal (poly I:C and CpG-ODN, TLR-7 and -9 agonists respectively). We found that PCEP and PCPP alone did not significantly upregulate *nod1, nod2* and *nlrp3*, nor genes for cell activation markers such as CD80 and CD86. However monocytes cultured with the combination of CpG-ODN, Poly I:C and PCPP appeared to moderately express IL-18, CD80 and CD86.

The secretion of pro-inflammatory cytokines from cultured monocytes was determined with Enzyme Linked Immunosorbent Assays (ELISA). It was found that IL-1β was secreted in significantly higher quantities in the supernatant of cells stimulated with both polyphosphazene and TLR ligands, as opposed to those cultured with polyphosphazene alone. Assays for IL-18, IL-6, IL-10 and IL-12 did not detect a significant presence of these proteins in the supernatant. Furthermore, we found that a soluble caspase inhibitor did not significantly reduce the production of IL-1β by monocytes, and was likely attributable to cell death at high concentrations.

Taken together, these results suggest that porcine monocytes, B cells and dendritic cells express elevated levels of the NLRs as compared to T cells. Additionally, areas of the respiratory tract appear to express increased levels of these receptors relative to mucosal and lymphoid tissues of the gastrointestinal tract. Neither PCPP or PCEP alone were capable of inducing significant production of IL-1β or IL-18 by cultured monocytes, however stimulation of these cells with a combination of CpG-ODN, poly I:C and polyphosphazene resulted in the secretion of IL-1β.

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Ag	antigen
APC	antigen presenting cell
ATP	adenosine tri-phosphate
BDC	blood dendritic cell
BSA	bovine serum albumin
BRV	bovine respiratory virus
BM	bronchial mucosa
BLN	bronchial lymph node
CIITA	human gene which encodes a protein called the class II, major histocompatibility
CARD	caspase activation and recruitment domain
CCR	chemokine receptor
CD14	cluster of differentiation molecule 14
CD80	cluster of differentiation molecule 80
CD86	cluster of differentiation molecule 86
CD172	cluster of differentiation molecule 172
CD21	cluster of differentiation molecule 21
CD3	cluster of differentiation molecule 3
cDC	conventional dendritic cell
cDNA	complementary deoxyribonucleic acid
CI	caspase inhibitor
CLRs	C-Lectin type receptors
CoS	Co-stimulatory molecule
CpG-ODN	CpG-Oligodeoxynucleotides
CrD	Crohn's disease
Ct	cycle threshold
CTL	Cytotoxic T-lymphocyte
DAMPs	danger associated molecular patterns
DC	dendritic cell
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid FCAS
ELISA	enzyme-linked immunosorbent assay
FHA	filamentous hemagglutinin
FIA	Freund's incomplete adjuvant
GW	gut wall
HBsAg	hepatitis B surface antigen
HEL	Hen egg lysozyme
HiB	*Haemophilus influenzae* type B vaccine

HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
HSV-2gD	herpes simplex virus type-2 glycoprotein
IDRs	innate defence regulator peptides
IFNg	interferon gamma
IgG2a	immunoglobulin 2a
IL-1β	interleukin-1 beta
IL-1	interleukin 1
IL-6	interleukin 6
IL-12	interleukin 12
IL-18	interleukin 18
IPV	inactivated poliovirus vaccine
ISCOMs	immune stimulating complexes
L	lung
LPS	lipopolysaccharide
LRR	leucine rich region domain
LTA	lipotechoic acid
LY6C	lymphocyte antigen 6C
MACS	magnetic activated cell sorting
MALLS	multi-angle light scattering
MDP	muramyl dipeptide
Meso-DAP	meso-diaminopimelic acid
MLN	mesenteric lymph node
MMR	measles, mumps and rubella vaccine
MPL	monophosphoryl A
MS	multiple sclerosis
MSU	monosodium urate
MWS	muckle wells syndrome
NACHT	nucleotide binding domain common to all NLRs
NF-κB	nuclear factor -kB
NK = natural killer cell	
NLRs	NOD-like receptors
Nlrp3	NACHT, LRR and PYD containing protein -3
NM	nasal mucosa
NO	nitric oxide
NOD	nucleotide oligomerizing domain
Nod1	nucleotide oligomerizing domain containing protein -1
Nod2	nucleotide oligomerizing domain containing protein -2
NOMID	neonatal onset multisystem inflammatory disease
PAMPS	pattern-associated molecular patterns

PBSA	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCEP	poly[di(sodium carboxylatoethylphenoxy]phosphazene
PCPP	poly[di(carboxylatophenoxy]phosphazene
pDC	plasmacytoid dendritic cell
PGN	peptidoglycan
Poly I:C	polyinosinic:polycytidylic acid
PP	polyphosphazene
PRRSV	porcine reproductive and respiratory syndrome virus
PRRs	pattern recognition receptors
PRV	pseudorabies attenuated virus
PSA	porcine serum albumin
PspA	pneumococcal surface protein A
PTd	pertussis toxoid
RA	rheumatoid arthritis
RLRs	RIG-Like receptors
RNA	ribonucleic acid
RPL-19	ribosomal protein L-19
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
RT	room temperature
SLE	systemic lupus erythematous
SWC1	swine workshop cluster number 1
SP	spleen
TAE	tris-acetate
TLR	toll-like receptor
T2D	type-2 diabetes
TLR	Poly I:C and CpG-ODN
TLR-MDP	Poly I:C, CpG-ODN and MDP
TLR-PCPP	Poly I:C, CpG-ODN and PCPP
TLR-PCEP	Poly I;C, CpG-ODN and PCEP
TNF	tumor necrosis factor
UC	ulcerative colitis
UVB	ultraviolet B
WHO	World Health Organization
X:31	influenza X:31 antigen

1.0. LITERATURE REVIEW

1.1. A brief introduction to vaccines

Vaccination is the most effective method of preventing infectious diseases (World Health Organization (WHO, 2013). Every year, countless lives are saved due to immunization against many highly infectious and deadly diseases. Historically, observations that the immune system expressed a capacity for memory and protection against specific pathogens were what led to the development of vaccines. Although the concept of vaccination had been around for centuries, it was not actively described until 1796 by the English physician, Dr. Edward Jenner. The term vaccination derives from the latin 'vacca' meaning cow as it was observed during the smallpox epidemic that milk maids who were afflicted with cow pox did not acquire smallpox disease. Jenner remarked that cowpox not only protected against small pox, but that protection could also be passed from one individual to another by immunization. Using infectious material extracted from cowpox lesions on the hands and arms of a young milkmaid, Sarah Nelms, Jenner immunized an 8-year old James Philips. Philips consequently developed symptoms of smallpox, but did not acquire the disease following subsequent inoculation with infectious small pox material (Riedel, 2005). Nearly 200 years after Jenner's work, smallpox was declared to be globally eradicated following a successful vaccination campaign. Furthermore, other infectious diseases such as polio, measles, mumps and rubella have also been significantly hindered by vaccines, therefore demonstrating that vaccines are an important and invaluable public health tool.

1.1.1. Types of vaccines

The development of an effective vaccine must consider the nature of the antigen, the correlates of disease protection, and the possible requirement of an adjuvant. Adjuvants are natural or synthetic compounds which enhance or modulate the immunogenicity of weak antigens, and are generally considered to be critical components of both whole killed or subunit vaccines (Mutwiri et al., 2008). A vaccine may be categorized as either: live-attenuated, inactivated, toxoid, subunit, conjugated or a DNA vaccine based upon its composition and function within the body (National Institute of Health, 2013). Live attenuated vaccines are generated by the passage of pathogenic organisms through a series of cell cultures or animal embryos. These vaccines contain live, replicating microbes that have acquired mutations which render them less virulent than their pathogenic forms. A key feature of live-attenuated vaccines are their ability to mimic a natural infection. Often, live-attenuated vaccines will confer lifelong immunity and elicit strong cellular and antibody responses after one or two immunizations, which make them ideal for areas of minimal vaccine coverage. However, due to the live nature of these vaccines, they possess the potential to revert back to a disease-causing agent. For this reason, live-attenuated vaccines may not be administered to certain age and health status groups. Examples of live-attenuated vaccines include those for measles, mumps and rubella (MMR), rotavirus, varicella (chicken pox), and yellow fever (Zust and Dong, 2013)

Chemicals, heat or radiation can be used to produce killed or inactivated vaccines. Such vaccines are more stable and safer than live vaccines. Due to their non-replicating nature, killed or inactivated vaccines are often weakly immunogenic and require the co-administration of an

adjuvant. Polio (IPV), hepatitis A and rabies, cholera and typhoid are examples of killed or inactivated vaccines (Lin and He, 2012).

Certain bacterial diseases such as whooping cough and staphylococcal associated endocarditis are caused by toxins produced by the bacteria. Toxoid vaccines contain a weakened or chemically modified form of the toxin. Certain toxoids such as pertussis toxoid, diphtheria toxoid, and tetanus toxoid are poorly immunogenic and require an adjuvant. Vaccines for diphtheria, tetanus and pertussis have been engineered from inactivated toxins.

Subunit vaccines contain highly purified protein antigens or epitopes, which stimulate the immune system. As subunit vaccines do not contain the full microorganism, they are often poorly immunogenic and require the addition of an adjuvant. Subunit vaccines are generally considered to be safe and efficacious, though they commonly require multiple immunizations to sustain immunity. Examples of subunit vaccines include those for hepatitis B, influenza, pertussis, meningococcus, pneumococcus and *haemophilus influenza* type B (Coffman et al., 2010).

Bacteria such as *Streptococcus pneumoniae* contain polysaccharide coats, which are poorly immunogenic and provide a mechanism for the pathogen to evade the immune system. In order to induce an immune response to such microbes, they must be conjugated to immunogenic antigens. Examples of conjugated vaccines include the *Haemophilus influenzae* type B (HiB) and pneumococcal polysaccharide vaccines (Berger, 1998).

DNA vaccines have demonstrated great promise in the protection against many infectious diseases and for the therapeutic treatment of cancer. These vaccines are based on the delivery of plasmids encoding genes for immunogenic proteins to targeted cells (Davis, 1997). Generally, these vaccines are inexpensive to produce and are considered to have a good safety profile;

however they are often poorly immunogenic and require specific methods, or adjuvants, to enhance their delivery to a targeted cell type or tissue. Currently, DNA vaccines are available for equine West Nile Virus and canine melanoma (Davis et al., 2001; Bergman 2007). Recombinant vector vaccines are similar to DNA vaccines, but use an attenuated virus or bacteria to introduce microbial DNA into a host cell. Vector vaccines have the capacity to actively invade and replicate within a host cell and produce antigenic proteins, which can tailor a specific immune response, making them fairly immunogenic (Davis et al., 2001). Adenovirus, vaccinia virus and attenuated poliovirus have been used in this manner (Mandl et al, 2001).

Dendritic cells play a central role in the induction and regulation of the immune response at many locations within the body (Coombes and Powrie, 2008). Recently, personalized vaccines have been investigated for the therapeutic treatment of cancer (Fioretti et al., 2010). This method involves removing dendritic cells from a patient and pulsing them with antigen prior to reinstating them back in to the host. It is thought that these cells may prompt a specific cytotoxic T lymphocyte response, which would guide the immune system to fight against certain tumor antigens (Berzofsky et al., 2004). Unfortunately, the extensive cost and time required to produce these vaccines severely limit their widespread use.

1.2. Adjuvants

The major goal of vaccination is to safely induce protective immune responses to a specific pathogen or disease. The concept that immune responses could be improved by the addition of certain compounds has been around for approximately 100 years (Leroux-Roels, 2010). Gaston Ramon, a French veterinarian and biologist, was the first to describe an adjuvant as 'a substance that is used in combination with a specific antigen, results in a more robust response than the antigen alone' (Ramon, 1924). A couple of years later in 1926, Alexander Glenny and colleagues reported that the precipitation of diphtheria toxin and aluminum potassium sulfate, otherwise known as Alum, was able to greatly enhance the toxin-specific antibody response in guinea pigs relative to untreated toxin (Glenny et al., 1926). Although the precise mechanisms underlying the adjuvant activity of Alum have not yet been determined, alum-based salts remain as one of the most widely used adjuvant in vaccine formulation, and until recently, was the only adjuvant approved for use in humans.

1.2.1. Types of adjuvants:

Adjuvants can be classified into two broad categories based on their presumed mechanism of action (Eng et al., 2010). Generally, adjuvants are either natural or synthetic compounds, which can act as either delivery vehicles or immunomodulators for antigens in a vaccine (Aguilar and Rodriguez, 2007). Delivery vehicles function to carry and retain antigens in close proximity to the lymphoid tissues (depot effect) (Marrack et al., 2009). Alum, liposomes, emulsions, immune stimulating complexes (ISCOMs) and numerous investigative particulate adjuvants are thought

to exert their effect by this mechanism (Mosca et al., 2008; Cox and Coulter, 2007). Immune modulatory adjuvants act by stimulating the innate immune response to enhance the secretion of cytokines and chemokines, which promote cell recruitment, cell maturation and antigen presentation to effector cells (Cox and Coulter, 2007). Such adjuvants include: CpG-oligodeoxynucleotides (ODN), muramyl dipeptide and monophosphoryl A (MPL).

Recent advances in the understanding of both the innate and adaptive responses have elucidated various mechanisms which allow scientists to tailor the outcome of the immune response to a vaccine using a variety of pattern recognition receptors (PRRs), such as the Toll-Like Receptors (TLRs), NOD-Like Receptors (NLRs), RIG-Like Receptors (RLRs) and C-Lectin Type Receptors (CLRs). These receptors function in the specific recognition of conserved microbial associated molecular patterns (MAMPs) and danger associated molecular patterns (DAMPs) (Rappuoli and Bagnoli, 2012). Furthermore, they are known to act cooperatively to activate a variety of transcription and translation factors involved in specific immunological pathways (Kawai and Akira, 2011). Adjuvants which stimulate PRRs can be used for many different applications. For example, the addition of an adjuvant to a vaccine may enhance the protective immune response and promote memory to a specific antigen (Cox and Coulter, 1997). The incorporation of an adjuvant in a vaccine formulation may also provide valuable antigen sparing properties by significantly decreasing the antigen dose requirement and contributing to efficient manufacturing and cheaper production costs. Lastly, adjuvants may also be used to reduce the number of immunizations needed to maintain a protective immune response by enhancing the amplitude of the immune response and prolonging the duration of immunity (Polewicz et al., 2011)

1.2.2. Mechanisms of action of adjuvants.

The mechanisms by which adjuvants exert their effect are not well understood. An adjuvant may act to sustain the physical persistence of the antigen at the site of injection and facilitate the prolonged exposure of antigen to the immune system (depot effect)(Cox and Coulter, 2007). Delivery system adjuvants may act to transport antigen to the APC environment or bystander cells, protect the physical and structural integrity of the antigen, or influence the presentation of antigen peptides to specific immune cells (Cox and Coulter, 2007). Immunostimulatory-type adjuvants may enhance the migration, maturation and expression of co-stimulatory molecules which improve the B and T cell response to antigen (Cox and Coulter, 2007)(McKee et al., 2007).

Studies of the mechanism of action of alum, the oldest and most widely used adjuvant have produced conflicting results. It was originally thought that the adjuvant effect of alum was due to the formation of a depot. Several experiments have also shown that alum may induce the rapid influx of neutrophils and eosinophils to the site of injection (McKee et al., 2007). Recent *in vitro* experiments have shown that alum can activate the NLRP3 inflammasome to produce pro-inflammatory cytokines which may be attributed to the adjuvant effect of alum (Colegio et al., 2008). However these results have not been observed *in vivo* and DCs which have been directly exposed to alum do not fully upregulate co-stimulatory molecules and do not produce Th-1-type cytokines (McKee et al., 2007).

Another widely used adjuvant MF59 is a potent adjuvant for recombinant proteins, particulate antigens and protein polysaccharide conjugates (O'Hagan et al., 2012). The immunological effects of MF59 are currently thought to result from the creation of a transient immunocompetent

environment at the site of injection (O'Hagan et al., 2012). It has been proposed that tissue resident cells, such as macrophages and muscle cells may respond to MF59 by producing cytokines, chemokines and other factors which enhance the migration and recruitment of immune cells, such as neutrophils, monocytes, eosinophils and dendritic cells to the site of injection (Calabro et al., 2011). As a result, an increased number of immune cells at the site of injection facilitate the interaction between an APC and antigen, which is thought to result in more efficient transport of antigen to the lymph nodes and T cell priming. Furthermore, the resulting amplification of immune cells in contact with MF59 may result in greater numbers of antigen specific effector and memory cells, enhanced differentiation of DCs, antibody secretion and ultimately greater vaccine potency (O'Hagan et al., 2012).

1.2.3. CpG-ODNs

CpG oligodeoxynucleotides (ODN) are potent activators of the innate immune response and have shown great potential as a vaccine adjuvant in many animal models and human clinical trials of infectious disease, allergy and cancer (Mutwiri et al., 2004). Synthetic CpG-ODNs motifs are comprised of central unmethylated C-G dinucleotides flanked by specific bases that closely mimic those found naturally in bacterial DNA (Mutwiri et al., 2004). Knock out studies performed in mice have shown CpG-ODN to signal through TLR-9, an endosomal PRR which functions as an upstream regulator for factors involved in the synthesis of a variety of pro-inflammatory cytokines and chemokines (Hemmi et al., 2000).

As compared to most conventional adjuvants, including alum, which is known to primarily induce a Th2-type immune response, CpG-ODNs are effective at enhancing the Th1 and an inflammatory type immune response characterized by the production of IgG2a, IL-1, IL-6, IL-12, TNF_a and IFN_g. This type of response is required for protection against certain infectious diseases, specifically those caused by intracellular microorganisms. For example, the co-formulation of CpG-ODN with porcine reproductive and respiratory syncytial virus (PRRSV), *Streptococcus suis* or pseudorabies attenuated virus (PRV) was shown to enhance the production of both IgG2 antibody and IFN_g, and switch the immune response towards protective Th1 phenotype (Linghua et al, 2006; Ming et al, 2013; Charerntantanakul, 2009).

The adjuvant effect of CpG-ODNs was shown to vary significantly between species, and is attributed to differences in the distribution of TLR-9 among various cell and tissue populations (Mutwiri et al., 2009). As such, some animal models may not accurately reflect the therapeutic capacity of CpG-ODNs in mammals. Recent Phase I- III clinical trials using CpG-ODN have shown this adjuvant to be well tolerated and not associated with any adverse injection site reactions (Bode et al., 2011).

Importantly, the versatility of CpG-ODNs is evident in studies of vaccine formulations containing multiple adjuvants. CpG-ODNs have been shown to work synergistically with other adjuvants including Alum, Freund's incomplete adjuvant (FIA), QuilA, Emulsigen, innate defense regulator peptides (IDRs) and polyphosphazene to enhance the magnitude, duration and quality of the immune response, often by promoting a mixed Th1/Th2 type response which may provide protection against a broad range of pathogens (Ioannou et al., 2003; Mutwiri et al., 2009). Furthermore, the co-formulation of CpG with other adjuvants did not compromise the

efficacy of the vaccine, and was proven to be a safe and effective modulator of the immune response in a number of animal studies.

1.2.4. Poly I:C

Polyinosinic: Polycytidylic acid (Poly I:C) are synthetic analogs of double stranded viral RNA. Similar to CpG-ODNs, Poly I:C mimics a molecular pattern associated with viral infection and functions to induce the transcription and translation of a variety of cytokines, chemokines and costimulatory molecules. Studies have shown that Poly I:C interacts with the endosomally expressed PRR, TLR-3 and cytoplasmic receptor MDA-5 to produce IL-6, IL-12, TNF_{α} and Type I IFNs which are potent inducers of DC maturation and B cell activation. In pigs, Poly I:C was demonstrated to significantly enhance the expression of cell surface molecules CD80, CD86 and CCR7 on both blood dendritic cells (BDCs) and monocyte derived dendritic cells (MoDCs), which is thought to play a critical role in mediating mature DC migration to the secondary lymphoid organs (Auray et al., 2010). Furthermore, the addition of Poly I:C was shown to enhance the antigen specific CD_8^+T cell cytotoxic and CD_4^+T cell humoral response to *Plasmodium falciparum* circumsporozoite protein (Tewari et al., 2010). Taken together, the immunostimulatory activity of various TLR-ligands, including CpG-ODNs and Poly I:C, make them desirable candidates for the development of novel vaccines.

1.2.5. Polyphosphazenes

Polyphosphazenes are a relatively new class of synthetic polymers, which have been shown to act as both an immunostimulatory type adjuvant and delivery vehicle for antigens in a vaccine (Garlapati et al., 2011; Mutwiri et al., 2007). Polyphosphazenes are comprised of high molecular weight chains that contain backbones of alternating phosphorous and nitrogen atoms with two organic side groups attached to each phosphorous that can be substituted to alter the chemical and physical properties of the polymer (Andrianov et al., 1998; Lakshmi et al., 2003).
Their inherent capacity to be biodegradable, permeable, hydrolytically unstable, result in non-toxic degradation products and easily synthesized make polyphosphazenes an attractive candidate for vaccine application (Andrianov et al., 2006; Mutwiri et al., 2007).

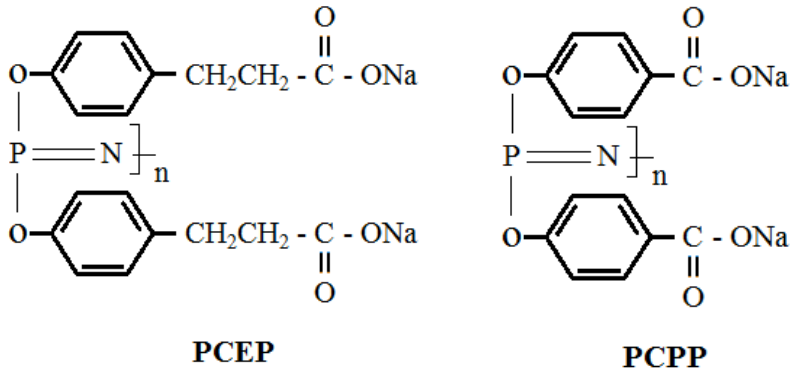


Figure 1. The chemical structure of the polyphosphazenes PCEP and PCPP.

Several studies have shown that the polyphosphazenes perform better than conventional adjuvants, both *in vivo* and *in vitro*. Specifically, animals co-vaccinated with antigen and polyphosphazene were shown to produce increased levels of chemokines (CCL2, CXCL-10), cytokines (IFN$_\gamma$, IL-1β,IL-4, IL-6, IL-12 and IL-18) and antigen specific Ig (IgG1, IgG2a), resulting in a more balanced Th1/Th2 immune response, and maintainance of a longer duration of protective immunity to a variety of bacterial, viral and non-microbial antigens including influenza X:31, tetanus toxoid, hepatitis B surface antigen (HBsAg), herpes simplex virus type 2 glycoprotein D (HSV-2 gD), bovine respiratory syncytial virus (BRV), pertussis toxoid (PTd), porcine serum albumin (PSA) and bovine serum albumin(BSA) (Mutwiri et al., 2007; Mutwiri et al., 2008; Gracia et al., 2011; Payne et al., 1997; Dar et al., 2012; Kovacs-Nolan et al.,2009; Polewicz et al, 2011; Eng et al, 2010; Eng et al, 2009).

A unique feature of the polyphosphazenes is their ability to form microparticles through a simple two-step method involving aqueous coacervation and ionic cross-linking. Importantly, these microparticles are thought to enhance the incorporation, stability, integrity and delivery of an antigen during vaccine formulation, processing and storage (Payne et al., 1995; Andrianov et al., 1998). Furthermore, the microparticle formulation of polyphosphazenes was shown to safely and effectively induce an antigen specific immune response in both the systemic and mucosal compartments of the body, thereby demonstrating an ability to overcome some of the challenges of targeted antigen delivery (Eng et al., 2009; Garlapati et al., 2011).

1.2.5.1. The adjuvant activity of polyphosphazenes

The most investigated polyphosphazene polyelectrolyte, poly[di(carboxylatophenoxy]phosphazene (PCPP) has proven to be a versatile and effective adjuvant in a number of studies. For example, aqueous PCPP was previously shown to promote the immune response to a diverse group of antigens including *Haemophilus influenzae* type b polyribosribotolphosphate (HiB), Hepatitis B surface antigen (HBsAg), HSV gD2 (Payne et al., 1997), and HIV envelope proteins (Lu et al., 1996). With the exception of HiB, a single dose of immunogen mixed with PCPP elicited persistently high antibody titers that lasted for at least 6 months (Andrianov and Payne, 1998) thus promoting vaccine longevity. Furthermore, PCPP was shown to be as effective as Freund's adjuvant at eliciting high serum IgG antibody to both influenza and tetanus toxoid antigens (Payne et al., 1998). PCPP admixed with influenza was also shown to improve haemagluttination inhibition, neutralize infectivity, and enhance IgM, IgG and IgG1 antibody titers by 10-fold relative to antigen alone (Payne et al., 1998).

A new generation of polyphosphazene, poly[di(sodium carboxylatoethylphenoxy)phosphazene](PCEP) was shown to have adjuvant activity that far surpassed PCPP in terms of quality and magnitude of the immune responses induced (Eng et al., 2010). Although they are similar in structure, PCEP was shown to significantly increase antibody titers of IgG1 and IgG2a and promote a mixed Th1 and Th2 type immune response when formulated with HBsAg, influenza and bovine serum albumin (Mutwiri et al., 2007; Mutwiri et al., 2008). As compared to the conventional adjuvant Alum, PCEP was shown to induce a 1000-fold higher immune response as measured by antigen specific antibody. Furthermore, a single immunization with PCEP and immunogen sustained antibody titers for at least 6 months with no

requirement for booster immunizations. Additionally, PCEP was shown to reduce the requirement for antigen by 25-fold without significantly altering the quality or magnitude of the immune response (Mutwiri et al., 2007) suggesting that PCEP may improve the cost of vaccination and provide strategies to improve vaccine coverage in various circumstances.

1.2.5.2. Polyphosphazenes modulate the quality of the immune response

Immunity to different infectious agents requires distinct types of immune response (Mutwiri et al., 2007). Protection against extracellular pathogens often require the induction of a humoral response controlled by increased levels of IgG and IgE antibody, while resistance to intracellular pathogens tend to involve a Th1-type immune response dominated by the production of IgG2a, cytokines IFNγ, TNF and cytotoxic T lymphocytes. Adjuvants can be a powerful tool to tailor the quality and type of immune response desired for a vaccine (Mutwiri et al., 2007). In studies with influenza antigen, both PCEP and PCPP were shown to significantly enhance the antigen specific IgG1 immune response, however PCEP was also demonstrated to promote antigen specific IgG2a production and enhance the Th1 and Th2 immune response through production of IFN$_{\gamma}$ and IL-4 (Mutwiri et al., 2007). This indicates that PCEP is capable of promoting broad-spectrum immunity while PCPP can be used to provide protection against extracellular pathogens. Furthermore, both PCEP and PCPP have demonstrated significant antigen sparing capacity making one-dose vaccines a real possibility (Polewicz et al., 2013).

1.2.5.3. Polyphosphazene as a mucosal adjuvant

The majority of infectious diseases are caused by pathogens, which colonize and invade mucosal surfaces such as the respiratory, gastrointestinal and genitourinary tracts. Pathogen specific secretory IgA at the site of infection may offer an effective means of protection for the host. Currently, most commercial vaccines administered via the parenteral route are not sufficient at inducing mucosal immunity. Mucosal immunization generally offers better protection, but is limited by the induction of oral tolerance and lack of a safe and effective mucosal adjuvant. Recently, studies evaluating PCEP and PCPP as mucosal adjuvants have shown promising results. For example, it has been shown that both oral and intranasal administration of influenza X:31 and PCEP resulted in significantly enhanced antibody titers in the nasal, lung and vaginal compartments as compared to immunization of antigen alone (Eng et al., 2010). The addition of PCEP was also shown to reduce the antigen requirement by 5-fold without compromising the quality or magnitude of the immune response (Eng et al., 2010). Similarly, mice immunized intranasally with PCPP and either pertussis toxoid (PTd), pneumococcal surface protein A (PspA) or Influenza virus strain A/Puerto Rico/8 (PR8), resulted in the significant production of antigen specific IgG and IgA antibodies at distal systemic and mucosal compartments (Shim et al., 2010). Furthermore, the intranasal administration of PCPP resulted in significant antibody titers in nasal, saliva, vaginal, and fecal washes (Shim et al., 2010). Co-formulation of antigen and PCPP may also promote the differentiation of B cells into antibody secreting plasma cells as it was shown that mice immunized intranasally with PTd and PCPP had significantly more PTd-specific IgA antibody secreting cells in the nasal passage, lung and sub-mandibular glands. Elevated levels of IgG antibody-secreting cells were also found in the nasal cavity and lung as compared to mice immunized with antigen alone (Shim et al., 2010).

Taken together these results suggest that polyphosphazenes are effective as mucosal adjuvants and may offer a safe and effective alternative to novel vaccine formulations.

1.2.5.4. The safety of polyphosphazenes

Many potential adjuvants fail to advance to clinical studies due to toxicity concerns (Eng et al., 2010). In a Phase I clinical trial of influenza vaccine in young and elderly adults, the addition of PCPP was shown to enhance serum titers to influenza strain, A/Johnnesburg/33/94 by almost 15-fold as compared to the unadjuvanted version of the vaccine which only produced a 3-fold increase. Furthermore, polyphosphazene PCPP was well tolerated with no evidence of adverse events at doses of up to 500ug (Bouveret et al., 1998). Additionally, Phase I and Phase II clinical trials of a vaccine incorporating PCPP and HIV-1 antigens did not result in either abscess at injection site, immune dysfunction, anaphylaxis, or allergy, whereas a vaccine formulated with Freund's complete adjuvant was associated with definable long-term adverse events (Gilbert et al., 2003). Polyphosphazenes have also been shown to be a safe and effective adjuvant in large animals at doses up to 1mg/animal. In a study which compared PCEP to the conventional adjuvant, Emulsigen®, polyphosphazene was demonstrated to result in fewer injection site reactions, including pain, swelling and delayed type hypersensitivity (Dar et al., 2012). Although these results emphasize the potential of polyphosphazenes to safely and selectively enhance the immune response, further detailed safety studies are required.

1.2.6. Novel adjuvant platform

Combination adjuvants are thought to exert their effect through the simultaneous stimulation of multiple PRR signaling pathways. Multiple adjuvant co-formulations have been shown dramatically enhance the magnitude and duration of the immune response to antigens present in a vaccine (Polewicz et al., 2011; Gracia et al., 2011; Kovacs-Nolan et al., 2009). A novel adjuvant platform, which was recently developed at VIDO-InterVac has demonstrated to safely and effectively induce both the humoral and cell mediated response when administered via the parenteral and mucosal routes. In studies of mice and larger outbred species, such as pig and cattle, the platform, which consists of the co-formulation of CpG-ODN or poly I:C, IDRs, and polyphosphazene (PP) was shown to synergistically enhance the production of antigen specific antibody and modulate the immune response through the secretion of chemokines and cytokines (Gracia et al., 2011; Polewicz et al., 2011; Mutwiri et al., 2008; Dar et al., 2012; Kovacs-Nolan et al., 2009).

Preliminary studies of the adjuvant platform evaluated the immune response to soluble, aqueous and microparticle formulation of these adjuvants in combination with pertussis toxoid antigen (PTd); a component of *Bordetella pertussis*, the causative agent of whooping cough, found that mice given a single subcutaneous immunization of polyphosphazene microparticles co-encapsulating CpG-ODN, IDR and PTd showed enhanced protection against challenge with *B. pertussis* than any component of the platform given individually, or in soluble or aqueous form. Furthermore, it was observed that mice immunized with the microparticle formulation exhibited a similar level of protection as those immunized with the leading vaccine for whooping cough, Quadracel™. However, in contrast to Quadracel™, which primarily induced a Th2-type response, MPs were observed to promote both a Th1 and Th17 immune response in the lungs as

evident by the enhanced secretion of TNF_{α}, IFN_{γ}, IL-6, IL-12, IL-17 and CCL2 (Garlapati et al., 2011). Importantly, the ability of MPs to induce a cell-mediated immune response distinguishes this adjuvant formulation from other conventional adjuvants, and may therefore contribute to greater protection from a variety of infection and disease (Garlapati et al., 2011).

A recent study performed by Gracia et al., 2011, evaluated the various classes of CpG-ODN, PP and IDRs combined with PTd *in vivo* . It was found that adult mice vaccinated with PCEP, IDR-HH18 (IWVIWRR-NH_2), CpG class-C (10101) and PTd resulted higher serum titers of antigen specific IgG2a and IgG1 than PTd alone, or any of the other adjuvant combinations tested. Furthermore, it was demonstrated that a single immunization with this combination of adjuvants, as compared to Quadracel™, resulted in an earlier onset of immune response and duration of immunity, which was reported to last greater than 22 months (Gracia et al., 2011). Similar experiments were performed in larger, outbred species using hen egg lysozyme (HEL) co-formulated with poly[di(sodiumcarboxylatophenoxy)phosphazene](PCPP), CpG and the antimicrobial peptide indolicidin (Kovacs-Nolan et al., 2009). Much like mice, it was found that young cows immunized with this combination of adjuvants also produced significantly higher serum IgG titers in comparison to other adjuvant formulations and the conventional adjuvant, Emulsigen®. Moreover, the adjuvant combination of CpG-ODN, PCPP and indolicidin was shown to induce a potent cell mediated immune response, and longer duration of immunity (Kovacs-Nolan et al, 2009).

The inhibitory effects of passively transferred antibody to neonates can often interfere with successful vaccination. Unfortunately, most neonatal morbidity and mortality due to infectious disease occur within the first few months of life, prior to an infant's first immunization at 2 months of age (Polewicz et al., 2011). Of particular significance is whooping cough which is

estimated to have an incidence of 20-40 million cases and to contribute to 200,000-400,000 infant deaths annually, despite the existence of several commercially available vaccines (Polewicz et al., 2011). Recently, an innovative experiment demonstrated that it was possible to prime the neonatal immune system to *Bordetella pertussis* in the presence of maternal antibodies (Polewicz et al., 2011). Briefly, pregnant mice and sows were vaccinated with varying concentrations of PTd. Offspring were then immunized by either parenteral or mucosal routes with co-formulations of filamentous hemagglutinin (FHA), PTd, CpG-ODN, IDR, and PCEP. It was found that MatAb interference in both pigs and mice could be overcome using a second booster immunization with the specific adjuvant combination, and that co-formulation of pertussis antigens with the novel adjuvant platform resulted in an earlier onset of immunity, superior IgG2a and IgA titers and a balanced Th1/Th2 immune response when compared to the Quadracel™. Importantly, this vaccine formulation contain significantly fewer antigens than Quadracel™ , and was proven to be safe and effective when delivered via the mucosal route. Taken together, these results indicate that the unique formulation of CpG-ODN, IDR and PP demonstrate a great potential for many diverse vaccine applications. Importantly, the versatility of this adjuvant platform to be delivered through parenteral and mucosal routes is advantageous from both a production and regulatory perspective and may provide a significant contribution to the improvement of global human and animal health.

1.3. The pig as a model for human infection and disease

1.3.1. The porcine immune system

Animal models are often used in research to predict therapeutic treatments for humans. A good animal model should be able to develop and survive the disease, produce multiple animals per gestation, and be large enough to provide an opportunity for multiple sampling (Meurens et al., 2012). Well-characterized methodologies and tools are available for study in mice and rats; however the anatomical particularities of rodents, including many immune parameters exhibit a less than 10% similarity to that of humans (Meurens et al., 2012). Furthermore, studies of toxicity, optimal vaccine dose and method of treatment may not accurately predict the therapeutic outcome in humans (Levast et al., 2012). Domestic pigs are ideal models for the study of human infectious disease. Similar to humans, the pig is an omnivorous, monogastric mammal that has shown to closely resemble humans in terms of anatomy, physiology and genetics (Meurens et al., 2012; Rothkotter, 2009). Domestic pigs have also been shown to contain the same immune cell populations present in humans, as well as a full set innate and adaptive immune responses. Like humans, pigs exhibit a high percentage of neutrophils in peripheral blood (50-70%) relative to mice (10-25%). Additionally, direct orthologs in pigs have been described for all human cytokines involved in the Th1, Th2, Th17 and Treg response, including interleukins 2-5,-10,-13 and interferon-γ (IFNγ) (Meurens et al., 2012). The expression of toll like receptors (TLR)-7 and -9 in porcine and human dendritic cells has also been shown to be restricted to plasmacytoid dendritic cells (pDCs), whereas mice express these TLRs in both pDCs and conventional dendritic cells (cDCs) (Auray et al., 2012). Furthermore, unlike mice, neither porcine nor human macrophages have been shown to produce nitric oxide (NO)

following LPS stimulation and IFNγ priming, thus demonstrating certain functional disparities in the innate immune response between species (Fairbairn et al., 2011; Meurens et al., 2012). Among the main physiological differences between humans and pigs is the existence of two distinct types of Peyer's patches, an inverted lymph node structure and an epitheliochorial placenta which prevent the transfer of passive immunity to the fetus during the intrauterine period (Rothkotter et al, 2009). Also, several innate defense regulator peptides which occur in humans are absent in the pig (Meurens et al., 2012; Rothkotter, 2009). Recently, the pig has been used as a model to study *B. pertussis*, influenza virus, RSV, *Mycobacterium tuberculosis*, *salmonella enteritica*, *shigella flexneri* and *clostridium difficile* (Polewicz et al., 2011; Levast et al., 2012) and has subsequently contributed to the development of novel vaccine formulations and therapeutics. Importantly, the extensive physiological and anatomical similarities of the pig provide researchers with a greater understanding and knowledge of the mechanisms that underlie both human and animal health.

1.3.2. Porcine monocytes and macrophages

Myeloid cells, such as monocytes and macrophages play an important role in the innate and adaptive immune response (Ezquerra et al., 2009). Both monocytes and macrophages are derived from a common haematopoetic stem cell in the bone marrow where their fate is determined by the expression of cell surface receptors. Ultimately, the strategic locations of both monocytes and macrophages contribute many crucial regulatory and effector functions at specific sites in the body (Ezquerra et al., 2009).

1.3.2.1 Monocytes

Monocyte subsets are divided based on their size, trafficking and expression of cell surface molecules (Shi and Pamer, 2011). In pigs, CD14 is expressed on monocytes, tissue macrophages and some granulocytes (Ezquerra et al., 2009) and can be used to isolate specific cell populations both *in vivo* and *in vitro*. CD14 has been shown to bind to a variety of bacterial and yeast products including lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), mycobacterial glycolipids and mannans. Furthermore, CD14 is thought to play a role in the recognition and phagocytosis of apoptotic cells. The ligation of CD14 has been shown to induce the secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-12, TNF_{α}), and the expression of surface molecules (CD172a, CD16, SWC1), which are involved in aspects of the innate and adaptive immune response in pigs (Ezquerra et al., 2009).

Monocytes have been shown to enhance resistance to a broad variety of microbial, viral, parasitic and fungal infections including: *Listeria monocytogenes*, *M. tuberculosis*, *Toxoplasma gondii*, *Plasmodium chabaldi*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Shi and Pamer, 2011). Currently, the precise mechanisms which mediate monocyte recruitment to specific tissue compartments have not yet been identified though are thought to occur through series of cascades involving trafficking, rolling, adhesion and transcellular migration. The production of chemokines such as CCL2 has been shown to facilitate the emigration of monocytes from the bone marrow, while the expression of ligands for chemokine receptors 1-5 (CCR1-5) and various integrins appear to promote the recruitment of monocytes to sites of inflammation (Shi and Pamer, 2011).

It is unclear what governs the differentiation of monocytes to become either macrophages or dendritic cells at sites of damage, though recent evidence suggests that the local cytokine environment plays an essential role in this process (Chomarat et al., 2003). Furthermore, it has not yet been determined how the overall number of mononuclear cells within an organ is controlled after homeostasis has been established (Shi and Palmer, 2011). Revealing the mechanisms which regulate these processes may provide for the development of innovative vaccines as well as therapeutic strategies to limit the pathology of various inflammatory diseases in which monocytes are implicated, including rheumatoid arthritis and artherosclerosis (Chomarat et al., 2003; Shi and Palmer, 2011; Huang et al., 2007).

1.3.2.2. Macrophages

Macrophages play an important role in antimicrobial defense, metabolism, tissue homeostasis and wound repair. They are strategically located throughout the body and function to provide immune surveillance, antigen presentation and immune suppression. Populations of macrophages are sub-divided based on their anatomical location, the expression of cell surface proteins, and their functional phenotype (Murray and Wynn, 2011). In the pig, macrophages can be distinguished based on the expression of F4/80 antigen, lymphocyte antigen 6C (LY6C) and chemokine receptors: CCR1, CCR2, CX3CR1. It is generally thought that tissue resident macrophages express $CCR2^{hi}$ and $LY6C^{low}$, while those expressing CX3CR1 and $LY6C^{hi}$ function in surveillance of the vascular endothelium (Fairbairn et al., 2011). The tendency for macrophages to exhibit a variety of phenotypes due to their plastic nature, combined with the

redundant expression of cell surface markers among myeloid cells ultimately contributes to a difficulty in isolating and identifying specific macrophage populations.

Macrophages exert their effect through a variety of pattern recognition receptors (PRRs), which allow the cell to effectively distinguish self from non-self, in addition to stimuli associated with cell lysis or tissue destruction. The Toll like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-1 (RIG)-like receptors (RLRs) and NOD-like receptors (NLRs) selectively activate transcriptional mechanisms that contribute to phagocytosis and secretion of cytokines such as tumor necrosis factor (TNF), IL-1, IL-6, IL-8 and IL-12. Much like the expression of cell surface proteins, the distribution of PRRs on, or within the cell, is not always conserved between species. For example, the disparity in CpG-ODN activity between rodents and pigs is thought to result from differences in TLR9 expression (Mutwiri et al., 2009). Various stimuli such as endotoxin and IFN_g have been shown to activate macrophages to phagocytose and kill microorganisms, secrete proinflammatory cytokines, and present antigen to T cells (Herbst et al., 2011). This is of particular importance throughout the gastrointestinal tract. The mucosa harbours the largest population of macrophages in the body, which are likely regulated in part by an environment rich in IL-10 (Murray and Wynn, 2011). The purpose of these cells is to maintain tolerance to food antigens and microbiota while recognizing and removing enteric pathogens. Unsurprisingly, the deregulation of intestinal macrophages often results in disease, or an inability to mount an effective immune response. Many viral and bacterial pathogens are known to replicate in porcine macrophages, including swine pox virus, porcine circovirus 2, porcine reproductive and respiratory syncytial virus (PRRSV), *Yersinia enterocolitica, Mycoplasma hypopneumoniae,* and *Actinobacillus pleuropneumoniae* (Fairbairn et al., 2011).

Recently, it was shown that *Salmonella typhimurium* infection induces the expression of the inflammasome gene, NOD2 in the Peyer's patches and gut wall of pigs (Meurens et al., 2009). Interestingly, it is thought that mutations, which render NOD2 unable to detect certain microbial stimuli, may contribute to the development of various inflammatory bowel diseases (Girardin et al., 2003). Furthermore, studies of PRRSV and Actinobacillus have suggested that disease susceptibility and pathology between individuals or specific breeds of swine may be correlated to the level of macrophage activation (Fairbairn et al., 2011). Taken together, it is evident that the potential of macrophages to detect and mount an appropriate inflammatory response following the recognition of specific microorganisms in the gut may provide a protective mechanism to various types of infection.

1.4. The inflammasomes

Innate immunity is the first line of defense against infection and functions to provide both an initial response against microorganisms and stimulate the adaptive immune response. Innate immune cells express a variety of germline encoded PRRs, which detect a broad range of pathogenic, endogenous and environmental stimuli. PRRs have been shown to mediate the induction of various intracellular signal cascades involved in the synthesis of cytokines, chemokines, adhesion molecules and costimulatory molecules (Schroder and Tschopp, 2010). The most widely characterized family of PRRs in the Toll-Like Receptors (TLRs), which are present on the plasma membrane and endosome of many cells. TLRs play a crucial role in the innate immune response through recognition of a variety of molecules, which are not expressed on mammalian cells. Furthermore, the identification of TLRs has played an important role in the development of many novel adjuvants and immunoenhancers (Leroux-Roels, 2010).

Recently, a large family of soluble cytosolic receptors have been identified which show similar characteristics to TLRs. The Nucleotide Oligomerizing Domain (NOD)-like receptors (NLRs) are constituently expressed intracellular proteins, which oligomerize into high molecular weight caspase activating platforms called “inflammasomes”. The inflammasomes have been shown to mediate the maturation and secretion of pro-inflammatory cytokines including IL-1β, IL-18 and IL-33 in response to a wide variety of stimuli including pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs; Martinon et al., 2009). Activation of the inflammasomes, both at a local and systemic level, carry the potential to significantly modulate the type of immune response and influence disease progression. As a means of regulating inflammasome activity, several theories have suggested that two signals may be

required for the activation of the inflammasome and subsequent production of IL-1β and IL-18 (Rathinam et al., 2012). First, the transcription of pro-IL-1β and pro-IL-18 require activation of the major transcription factor NF-κB by TLR ligation with an appropriate agonist such as LPS or TNF. A second signal, such as environmental stimuli or specific products of tissue damage or cell lysis, result in the cytosolic oligomerization of NLRs into a multi-protein complex which acts to recruit caspases to catalytically cleave pro-IL-1β and pro-IL-18 in to their mature, bioactive forms. NLRs consist of three main subfamilies, which are divided based on structure and function, these include: NODs (1-5, major histocompatibility complex II transactivator (CIITA)); NLRPs/NALPs (1-14); and NLRC4/IPAF (Schroder and Tschopp, 2010). Furthermore, each NLR is comprised of three regions: a carboxy-terminal leucine rich region (LRR), that is thought to function in ligand sensing and autoregulation; a central nucleotide binding domain (NACHT) which mediates protein-protein oligomerization, and an amino-terminal effector domain which controls caspase recruitment and activation (Martinon et al., 2009; Schroder and Tschopp, 2010). The molecular mechanisms which mediate the activation of NLRs are poorly understood, but thought to occur either through direct ligand binding, detection of ionic fluctuations following membrane perturbation or phagosomal destabilization resulting from engulfment of large particulate or crystalline structures (Schroder and Tschopp, 2010).

Many adjuvants have been shown to activate inflammasomes, but the role of the inflammasomes in adjuvant activity, specifically the adaptive immune response is not fully understood. NLRP3 has been implicated in the adjuvancy of Alum, Quil A/saponin and chitosan, which collectively demonstrate a potential to induce the capase-1 dependent secretion of IL-1β and IL-18, promote adjuvant mediated cell recruitment, and enhance antigen specific IgG titers *in vivo* (Li et al.,

2008; Eisenbarth et al., 2008; Kool et al., 2008). Conflicting results *in vivo* have demonstrated that antigen specific IgG production was not impaired in NLRP3 deficient mice following intraperitoneal injection of human serum albumin (HSA) in the presence of alum (Franchi and Nunez, 2008). However, NLRP3 appeared to be required for alum-mediated cellular recruitment, which may indicate that inflammasomes play a key role in the innate immune response to alum, but not the activation of the adaptive immune response.

1.4.1. NLRP3, NOD1 and NOD2

The most widely investigated inflammasome, NLRP3, has been shown to be activated upon exposure to a diverse group of microorganisms including *Staphylococcus aureus*, *Listeria monocytogenes*, *Neisseria gonorrhoeae*, influenza virus, adenovirus, *Candida albicans* and *Saccharomyces cerevisiae* (Schroder and Tschopp, 2010). Additionally, recent studies have identified NLRP3 activation in response to environmental stimuli: silica, asbestos, UVB irradiation (Cassel et al., 2008; Dostert et al., 2008; Feldmeyer et al., 2007) and specific products of tissue damage or cell lysis: nucleotides, extracellular ATP, extracellular glucose, monosodium urate (MSU), and amyloid B peptide (Schroder and Tschopp, 2010; Mariathasan et al, 2006; Halle et al., 2008). Furthermore, NLRP3 activation has been shown to occur in response to several endogenous signals such as low intracellular potassium (K+) concentration, the release of cathepsin B, or generation of reactive oxygen species (ROS) (Schroder and Tschopp, 2010; Kanneganti et al., 2006; Petrilli et al, 2007; Martinon et al, 2006).

NOD1 and NOD2 are the most characterized of the NOD signalsomes. Both contain similar LRR and NACHT regions, but differ in respect to their caspase recruitment and activation domains (CARD). Individually, NOD1 and NOD2 recognize specific highly conserved structural moieties of bacterial peptidoglycan (PGN). NOD1 has been shown to detect meso-diaminopimelic acid (meso-DAP), a component of the cell wall of most gram negative and some gram positive bacteria, while NOD2 recognizes muramyl dipeptide (MDP), the minimal structure of peptidoglycan and common component of all bacterial cell walls. Both meso-DAP and MDP are degradation products of bacterial cell wall released by intracellular or phagocytosed bacteria (Martinon et al, 2009).

NOD1 and NOD2 are considered to be important receptors in both antigen presenting cells and epithelial cells of the gut. Recent studies suggest that genetic mutations of NOD2 are associated with different inflammatory bowel conditions, including Crohn's disease. Moreover, the specificity of NOD1 and NOD2 for bacterial products may confer an advantage in the detection of intestinal bacteria and play a distinct role in the innate immune response at mucosal surfaces (Teshima et al., 2012)

1.4.2. Interleukin 1-beta

IL-1β is an important inflammatory mediator, which acts on lymphocytes to reinforce the adaptive immune response (Sims and Smith, 2010). It is mainly produced by monocytes and macrophages in response to pro-inflammatory stimuli, and functions to stimulate a wide range of biological processes. For example: IL-1β is thought to contribute to the maturation and proliferation of B cells, the expansion, differentiation and survival of T cells, as well as enhance

the growth factor activity of fibroblast cells (Sims and Smith, 2010; Zucali et al., 1986). Because it's highly pyrogenic nature, the production of IL-1β is tightly regulated at both the transcriptional and translational level. Inflammatory stimuli induce the expression of the inactive, 31 kDA pro-form of IL-1β within the cytosol, and cytokine release is controlled by the inflammasome protein unit which mediates the caspase dependent processing of IL-1β (Schroder and Tschopp., 2010). A broad spectrum of human diseases are caused by mutations which control the generation of active IL-1β. These include familial cold auto-inflammatory syndrome (FCAS), Muckle Wells Syndrome(MWS), neonatal onset multisystem inflammatory disease (NOMID), rheumatoid arthritis(RA), type II diabetes (T2D), and Crohn's Disease (CrD) (Schroder and Tschopp, 2010; Sher et al., 1995).

1.4.3. Interleukin-18

Interleukin-18 (IL-18) is a recently described cytokine that shares structural and functional similarities to IL-1β. IL-18 is primarily produced by macrophages, dendritic cells and epithelial cells; though its expression has also been reported in microglial cells, articular chondrocytes, keratinocytes, and synovial fibroblasts (Gracie et al., 2003; Foss et al, 2001). Similar to IL-1β, IL-18 is translated as an inactive pre-protein that relies on caspase-1 for cleavage in to its mature form. Studies of IL-18 have demonstrated it to be an important regulator of the innate and acquire immune response (Foss et al., 2001). Generally, IL-18 is described as a Th1 promoting cytokine, however in the presence of specific stimuli, IL-18 may induce the production of Th2 cytokines by both naïve and Th1 polarized cells (Sims and Smith, 2010). The

immunomodulatory properties of IL-18 make it an important contributor to the clearance of a broad range of viruses, intracellular bacteria, fungi and protozoa. IL-18 has been shown to work synergistically with IL-12 to activate both B cells and natural killer cells to produce IFN_{γ}, thereby down-regulating the humoral immune response and enhancing both antiviral and cytolytic activity. Furthermore, IL-18 has been shown to promote the secretion of IL-3, IL-5, IL-6, IL-13 and TNF_{α} by mast cells, and IL-4 and IL-13 by basophils in the presence of a second stimulus (Sims and Smith, 2010). Regulation of IL-18 occurs at both the transcriptional and translational level, and is further controlled at the post-modification level by a specific soluble binding protein which prevents IL-18 from interacting with its cognate receptors (Sims and Smith, 2010). Unlike IL-1β, IL-18 has been shown to be constitutively expressed within the cytoplasm of the cell and deregulation of its secretion is thought contribute to the pathological inflammation underlying a number of chronic human diseases. Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), diabetes, rheumatoid arthritis (RA), asthma, cardiovascular disease, Ulcerative colitis and Crohn's disease are examples (Sims and Smith, 2010).

2.0 HYPOTHESIS

I hypothesize that the stimulation of porcine monocytes with polyphosphazene PCEP or PCPP will result in the production of pro-inflammatory cytokines IL-1β and IL-18.

3.0 OBJECTIVES

1. Determine the patterns of expression of NOD-Like Receptors: NOD1, NOD2 and NLRP3 in porcine mucosal and lymphoid tissues, as well as porcine immune cells; T cell, B cell, monocyte and dendritic cells.

2. Characterize the activation of NOD1, NOD2 and NLRP3 in porcine monocytes in vitro via known NLR agonist muramyl di-peptide (MDP)

3. Determine whether polyphosphazenes: poly[di(carboxylatophenoxy)-phosphazene](PCPP), or poly[di(sodium carboxylatoethylphenoxy)-phosphazene](PCEP) signal through NOD1, NOD2 or NLRP3 to induce the production of pro-inflammatory cytokines IL-1β and IL-18.

4.0 MATERIALS AND METHODS

4.1 Animals

Six-to-eight week old Dutch Landrace pigs purchased from Prairie Swine Centre (University of Saskatchewan) were used in this study. All experiments were conducted in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

4.2 Polyphosphazene

PCPP and PCEP were synthesized by Idaho National Laboratory (Idaho Falls, ID, USA) using methods previously described by Andrianov et al., 2004; Andrianov et al.,2006. In summary, PCPP was synthesized from its hydrolytically unstable macromolecular precursor PDCP using sodium propyl paraben nucleophilic reagent dissolved in diglyme with propyl 4-hydroxybenzoate as a cosolvent. 85g of propyl paraben (0.48mol) was dispersed in 21mL of diglyme and the dispersion was heated with constant stirring until melted (110^0c). 96g of sodium propyl paraben (0.48mol) was then added to the melt and heating was continued until a clear solution was formed. The solution is then diluted with 190mL of diglyme and added to the three neck reaction flask charged with 130mL of 0.2M polydichlorophosphazene solution in diglyme while stirring. The reaction mixture was refluxed for 2h under nitrogen and then colled for 95^oC. 141mL of aqueous 13 N potassium hydroxide solution (1.8 mol) was slowly added with vigorous stirring to the reaction mixture to bring about hydrolysis and the subsequent precipitation of PCPP. 20mL of water was then added to facilitate efficient phase separation. The liquid organic layer was decanted and the precipitate was dissolved in 300mL of 15% (w/v) aqueous sodium

chloride solution and then precipitated by the addition of 600mL of deionized water. The aqueous layer was decanted, the precipitate dissolved in 150mL of deionized water, and finally precipitated by the addition of 150mL of ethanol. The PCPP precipitate was filtered and dried. The purity of the polymer was determined by HPLC analysis, Karl Fisher titration and elemental analysis. The polymer structure was verified by 31P and 1H NMR. (Andrianov et al, 2004). PCEP was prepared by reacting a solution of sodium salt of methyl 3-(4-oxyphenyl)propionate with polydichlorophosphazene in diglyme at 120^0C for 10 hours. The resulting PCEP polymers were then hydrolyzed using aqueous potassium hydroxide at 85^0C. Polymer was recovered by precipitating in sodium chloride solution and then ethanol in its salt form. PCEP polymers were confirmed by permeation chromatography, which was configured as follows: Waters 600 HPLC pump, Waters 717 plus Autosampler, an Ultra-hydrogel Linear column, a multi-angle laser light scattering (MALLS) detector (DAWN DSP-F, Wyatt Technology, Santa Barbara, CA), a Waters 996 Photo Diode Array detector, and a Waters 410 refractive index detector (Waters, Milford, MA). Phosphate buffered saline (PBS, pH7.4) containing 5% acetonitrile was used as a mobile phase. Polyphosphazene PCEP and PCPP were designed and synthesized by Parallel Solutions Inc. (Cambridge, MA). Aqueous solutions of both polymers were stored at room temperature in the dark, and were found to retain activity for over several months under these storage conditions. Batches of polyphosphazenes were tested and found to have endotoxin levels that were below 0.034ng/mL as assessed by Limulus Amebocyte Lysate assay (Biowhittaker, Walkersville, MD, USA). Following synthesis, endotoxin levels were assessed using a Limulus Ameobocyte Lysate assay (Biowhittaker, Walkersville, MD, USA) and determined to be less than 0.034ng/mL. Both polymers were dissolved in Dulbecco's phosphate buffered saline (PBS) (Gibco, NY, USA) and stored at RT in the dark.

4.3 Gel electrophoresis to determine amplified PCR product

To determine whether the amplified PCR products for *nod1, nod2, nlrp3, cd80, cd86,* and *il-18* were of correct size, 1 gram of agarose (Sigma-Aldrich) was dissolved in 49mL of ddH_20. Subsequently, 1mL of 50x Tris-acetate and ethylenediaminetetraacetic acid (EDTA) buffer (TAE) and 5uL of ethidium bromide solution (10mg/mL) were added. The solution was then poured in to a gel tray and allowed to solidify before being transferred to an electrophoresis chamber. 1x TAE buffer was used to fill the chambers. 10uL of each of PCR product was mixed with 2uL of loading dye (Thermo Scientific). For reference, 5uL of 100bp DNA ladder (MBI Fermentas) was added to a separate well for size determination. The gel was then run at 65-90 volts for approximately 1hour.

4.4 cDNASynthesis

cDNA was generated from the Invitrogen SuperScript III Platinum Two Step qRT-PCR kit with SYBR Green and followed manufacturer's guidelines. To quantify the relative gene expression, immune cell populations ($CD_3^{+/-}$, $CD_{14}^{+/-}$, $CD_{21}^{+/-,}$ and $CD_{172}^{+/-}$) were normalized to 500ng and tissue samples were normalized to 1000ng of RNA. Cultured monocytes were normalized to 8ng in samples that yielded RNA concentrations above 8ng/uL. To maximize enzyme efficiency, smaller concentrations of RNA were not diluted. cDNA was synthesized following 25 minutes of mixing at 25◦C (annealing), 50 minutes incubation at 42◦C (synthesis), 5 minutes at 85◦C (termination) and then stored on ice at -20◦C.

4.5 RT-qPCR Assay

Each reaction for each RT-qPCR consisted of 12.5uL Platinum SYBR Green qPCR supermix-UDG, 0.5uL of 10uM each primer, 2uL of cDNA and 9.5uL of ddH_2O for a final volume of 25uL. All samples were run in duplicate. Primer sequences were designed using Clone Manager (Sci-Ed. Cary, New York, USA) and data was obtained using Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA). mRNA was quantified following these conditions: 95◦ for 1 min, 45 repeats of 15 seconds at 95◦ (denaturation), 30 seconds at 60◦ (annealing), and 30 seconds at 72◦ (elongation), with 41 repeats of 10 seconds of 55◦ with 1◦ temperature change. The melt curves were used to evaluate the specificity of the reaction and the geometric mean of the cycle thresholds (Ct) were used to calculate the relative expression of NLR genes. Relative gene expression was quantified using the ΔCt method, while the fold of increase of expression was calculated using $2^{-\Delta\Delta Ct}$. Results were normalized to ribosomal protein L-19 (RPL-19). Ct values above 34 were not used in this study.

4.6 Isolation of Immune Cell Populations:

Positive selection for magnetic activated cell sorting (MACS) was used to isolate CD_3^+ T cells, CD_{14}^+ monocytes, $CD_{14}^-CD_{21}^+$ circulating B cells and $CD_{14}^-CD_{172}^+$ blood dendritic cells (BDC) from PBMCS. 1 liter of blood was taken from each of 6 pigs. PBMCs were isolated using a 60% FICOLL-PAQUE® Plus gradient (GE Healthcare, Uppsala Sweden). 1 $x10^8$ PBMCs were fractioned for T cell separation using 1uL of anti-CD3 antibody per 10^7 cells. On the remaining PBMCs, negative selection LD columns (Miltenyi Biotec) were used for CD_{14} separation. The

negative fraction of CD_{14} cells were used to isolate CD_{172}^{+} BDCs and CD_{21}^{+} B cells. $CD_{14}^{-}CD_{21}^{+}$ B cells were isolated using anti-CD_{21} antibody (Serotec) and rat anti-mouse IgG1 microbeads (Miltenyi Biotech). $CD_{14}^{-}CD_{172}^{+}$ BDCs were obtained by similar method using anti-CD_{172} and rat anti-mouse IgG1 microbeads (Miltenyi Biotech) . Both populations were acquired by positive selection using LS columns (Miltenyi Biotech). To isolate CD_{3}^{+} T cells, PBMCs were incubated for 20 minutes with anti-CD3 antibody (Miltenyi Biotec and rat anti-mouse immunoglobulin G1 (IgG1) Microbeads (Miltenyi Biotec) before being run through a positive selection LS column (Miltenyi Biotech). From each isolated sample, 10^7 cells were kept and suspended in TRIzol Reagent (Invitrogen) then stored at -80◦ for further RNA extraction.

4.7 RNA Isolation from Mucosal and Lymphoid Tissues

To isolate RNA from the tissues, segments of approximately 3mm x 3mm were surgically excised from the bronchial mucosa, nasal mucosa, lung, bronchial lymphnode, Peyer's patches, spleen, gut wall and mesenteric lymphode of each of 4 euthanized pigs. Specimens were collected in to cryotubes then snap frozen in liquid nitrogen. Tissues were then homogenized in 1mL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) using silica beads. Samples were then centrifuged at 1000x rcf for 10 minutes. In a fresh eppendorf tube, homogenates were combined with 200uL of Chloroform (Sigma-Aldrich) and allowed to sit at room temperature for 3 minutes. Samples were then centrifuged at 12,000g for 10 minutes at 4^0C. The aqueous phase was carefully collected and 500uL of isopropanol (Sigma-Aldrich) was added and allowed to incubate for 5 minutes at room temperature prior to applying to a Qiagen mini-column (Qiagen RNeasy, Missisauga, Ontario, Canada). Samples were then centrifuged for 15 seconds at 8,000g

and the columns were washed with 350uL of RW1 buffer (Qiagen) as per the manufacturers guidelines. Each column was incubated for 15 minutes with 80uL Rnase-Free Dnase-1 (Qiagen) at room temperature, then washed with 350uL of RW1 buffer and twice with 500ul RPE buffer (Qiagen) before being spun dry. 30uL of nuclease free water was added to elute RNA from each column. RNA quantification and purity was obtained using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON).

4.8 Monocyte Isolation

Syringes prepared with 5mL of 7.5% EDTA were used to collect approximately 500mL of whole blood from cardiac venipuncture of each 6-8 week old pig. Blood was then transferred to 50mL polypropylene centrifuge tubes and samples were spun at room temperature (RT) at 2500rpm for 30min with no brake. The buffy coat was carefully removed in to a fresh polypropylene tube containing 35mL of 0.1% EDTA in PBS using a Pasteur pipette coated with PBSA-EDTA. Cells were then layered on 60% Ficoll-Paque Plus gradient (GE Healthcare, Uppsala, Sweden) and centrifuged for 40 minutes at 400*x g* with no brake at RT. A fresh Pasteur pipette was used to remove the buffy coat at the gradient interface and cells were resuspended in 50mL of 0.1% PBSA-EDTA to wash. Samples were centrifuged for 10 minutes at 1200x rpm with brake to pellet the cells. Supernatants were then poured off and cells were washed 3 times further with PBS. Pellets from the same animal were combined in a low volume and PBMCs were counted using 1:1000 dilution of trypan blue.

PBMCs were then incubated with 95uL of MACS buffer (PBS supplemented with 5% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% EDTA and 5uL of human anti-CD_{14} microbeads (Miltenyi Biotec, Auburn, CA) per 10^7 cells, and gently mixed on nutator for 15 minutes at 4◦. Cells were then washed by adding up to 40mL of buffer and centrifuging for 300*x g* for 10 minutes. Positive selection for adherent CD_{14}+ monocytes was performed using LS Midi MACS^{TM} Columns (Miltenyi Biotec). Adherent cells were flushed using 5mL of MACS buffer, and then counted using 1:100x dilution of trypan blue to determine vitality and cell number.

Purified monocytes were plated at a concentration 1x10 ^{6} cells/mL in RPMI-1640 cell medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (SeraCare Life Sciences. Oceanside, California, USA), 0·5% mmβ-mercaptoethanol (Gibco), 10% of antibiotic/antimycotic (Gibco), 10% HEPES (Gibco), 10% MEM Non-essential amino acids (Gibco), in a volume of 500uL per well in a 48 well plate (Fisher Scientific) and incubated overnight at 37◦C and 5% CO_2.

4.9 Cell stimulation with TLR ligands and polyphosphazene

Cultured CD_{14}^{+} monocytes *in vitro*

Cell stimulation	LPS	MDP	PCEP	PCPP	TLR	TLR-MDP	TLR-PCEP	TLR-PCPP
LPS	+	-	-	-	-	-	-	-
MDP	-	+	-	-	-	+	-	-
PCEP	-	-	+	-	-	-	+	-
PCPP	-	-	-	+	-	-	-	+
Poly I:C	-	-	-	-	+	+	+	+
CpG-ODN	-	-	-	-	+	+	+	+

Table 1: Schematic of CD_{14}^{+} monocyte stimulation and activation *in vitro*. The columns represent the various treatment of monocytes . Groups represent the absence (-) or addition (+) of MDP, PCEP, PCPP, poly I:C or CpG-ODN to cells.

Resting MACS purified CD_{14}^{+} monocytes were stimulated in duplicate for 2 hours with either lipopolysaccharide (LPS) 1ug/mL (Escherichia coli 055:B5, Sigma-Aldrich, Oakville, Ontario, Canada) or a combination of TLR agonists: class A CpG-ODN 8954 (2.5ug/mL) (ggGGACGACGTCGTGggggggG) + poly I:C 50ug/mL (Sigma-Aldrich) to allow for transcription and translation of pro-IL-1β and pro-IL-18.

After two hours, 1ug/mL of muramyl di-peptide (Sigma-Aldrich), PCEP (25ug/mL) or PCPP (25ug/mL) was added to cells cultured with TLR ligands and allowed to incubate for 4 additional hours. Non-stimulated cells were cultured in supplemented RPMI-1640 cell media for the same time and served as control cells.

To study the gene expression and protein secretion, cells were centrifuged at 300*x g* for 10 minutes at 4° at 6, 12 and 24 hour time points. The supernatant was removed in to fresh eppendorf tubes and stored at -20◦. Cell pellets were resuspended in 500uL of TRIzol Reagent

(Invitrogen, Carlsbad, CA) and subsequently stored at -80◦ until RNA extraction could be performed.

4.10 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA for cytokine secretion was performed on monocytes co-cultured with TLR ligands and polyphosphazene. Samples were collected after 12 hours and 24 hours and spun at 300*x g* for 10 minutes at 4◦C. Supernatants were removed and stored at -20◦ for further use. IL-1β, IL-6 and IL-12 concentration were assayed using DuoSet ELISA development system (R&D Systems) and protocol was followed according to the manufacturer's instructions.

For IL-1β, polystyrene plates (Immunol II microtitre, Dynex Technology Inc., USA) were coated overnight with 2ug/mL of mouse anti-porcine IL-1β and incubated at room temperature (RT). Between each step, plates were washed 7 times in Tris-buffered saline containing 0.05% Tween 20 (TBST). To prevent non-specific binding, each well was incubated with 300uL of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hour at RT. 100uL of each supernatant sample was diluted 2-fold in Reagent Diluent (0.1% BSA, 0.05% Tween 20) and allowed to incubate for 2hours at RT. To detect protein, wells were then incubated with 100uL of 50ng/mL biotinylated goat anti-porcine IL-1β for 2 hours at RT. 100uL of 200x diluted streptavidin conjugated to horseradish-peroxidase was used for the detection of cytokine. Wells were incubated for 20 minutes at RT in the dark before adding 100uL of 3,3', 5,5' Tetramethylbenzidine (TMB)(Sigma-Aldrich) substrate solution for 20 minutes. 50uL of 2N-sulfuric acid (2N H_2SO_4) was used to terminate the reaction.

All samples were assayed in duplicate and the optical density was determined using a microplate reader (Molecular Devices, CA, USA) set to 450nm with a wavelength correction at 570nm. IL-1β concentration was determined by extrapolation of the standard curve generated by the serial dilution of *E. coli*-expressed recombinant mature porcine IL-1β.

4.11 Optimization IL-18 ELISA

To determine the optimal concentration of reagents required for IL-18 ELISA, a checkerboard titration (2-dimensional serial dilution) was performed for indirect ELISA. 96 well Immunol II microtitre plate (Dynex Technology Inc., VA, USA) coated with 100uL of 1ug/mL of anti-porcine IL-18/IL-1F4 antibody (R&D Systems Inc. Minneapolis, MN) in PBS, serial diluted across the plate for 6 columns. The plate was then wrapped and incubated overnight at room temperature. Plates were then washed 7 times in TBST and 300uL of 1% BSA in PBS was added to each well to block the plate for 1 hour at room temperature. The plates were then washed again and *E.coli*-expressed recombinant porcine IL-18 (R&D Systems Inc.) diluted in 0.1% BSA + PBS to final concentrations of 3000pg/mL, and 6000pg/mL, 12000pg/mL and 24,000pg/mL, 48,000pg/mL were added to each half plate and allowed to incubate for 2 hours at room temperature. Plates were washed and cytokine was detected using 1ug/mL of serially diluted biotinylated goat anti-porcine IL-18 antibody (R&D Systems Inc.). Wells were then incubated for 2 hours at RT. Detection was carried out using 100uL of streptavidin-HRP diluted (1:200) in 0.1% BSA in PBS and allowed to incubate at RT in the dark for 20 minutes. Plates were then washed and 100uL of 3,3' 5,5' tetramethylbenzidine (TMB) was added to each well and allowed to develop for 20-30 minutes at which point 50uL of 2N-sulfuric acid (2N H_2SO_4) was added to

the well. Optical density was obtained using a microplate reader (Molecular Devices, CA, USA) with wavelength set to 450nm and correction at 570nm. The optimal signal to noise ratio was calculated by dividing the average OD for different standard concentrations over the background noise.

4.12 Caspase Inhibitor Experiments

An assay to determine whether soluble caspase inhibitor could reduce the secretion of IL-1β in the supernatant of monocytes cultured with MDP or PCEP was performed. CD_{14}^{+} purified monocytes were isolated via MACS and plated in supplemented RPMI-1640 cell medium at a concentration of $1x10^6$ cells/mL in a volume of 200uL per well in a 48 well tissue culture plate. This volume chosen to minimize the amount of inhibitor required for the experiment. Caspase-1 Inhibitor (Caspase-1 Inhibitor VI, Calbiochem, EMD Biosciences, La Jolla, CA) was then added to the wells at concentrations of 25mM, 50mM, 75mM and 100mM two hours prior to stimulation with TLR ligands. Cell stimulation and activation were performed as previously described. Cultures were incubated for 24 hours before centrifuging for 10 minutes at 4°, 300x g to collect for supernatants. Samples were then stored at -20◦ for future use.

4.13 Statistical Analysis

Gene expression and antibody titers were compared using GraphPad PRISM™ 5 software, (GraphPad Software Inc., San Diego, CA, USA) and Excel (Microsoft®, Redmond, WA). The statistical difference between groups was determined using 1 way ANOVA of the ranked values

and compared using Tukey's multiple comparison test. Results were considered significant at $p < 0.05$. These methods allowed us determine whether differences existed between treatment groups, as well as between individual data points.

5.0 RESULTS

5.1 NLR gene expression in porcine immune cell populations

To determine the relative level of gene expression of the NOD-Like Receptors NOD1, NOD2 and NLRP3 in porcine immune cells, PBMCs were isolated from each of 4, six-week old pigs. Briefly, magnetic activated cell sorting (MACS) was used to positively select for CD_3^+ T cells, CD_{14}^+ monocytes, CD_{21}^+ B cells, and CD_{172}^+ dendritic cells via magnetically tagged monoclonal antibodies specific for CD3, CD14, CD21 and CD172 respectively. Both the positive and negative fractions from each cell separation were kept for analysis. Following RNA isolation, quantitative polymerase chain reaction (RT-PCR) was performed on the positive and negative fractions of MACS isolated cells (Figure 2). When normalized to ribosomal protein L-19 (RPL-19), we found that the relative expression of *nod1, nod2 and nlrp3* was significant in CD_{14}^+ monocytes, CD_{21}^+ B cells, CD_{172}^+ dendritic cells. Furthermore, we observed *nod2* to have the highest frequency of expression in porcine PBMCs (average relative gene expression of *nod1, nod2* and *nlrp3* in each cell population: Monocyte (0.003022); B cell (0.000722); and dendritic cell (0.001398); T cell (0.000055) relative to *nod1* and *nlrp3,* (*nod1:* monocyte (0.000513); B cell (0.000263); dendritic cell (0.000366); T cell (0.000002) (*nlrp3:* monocyte (0.002119); B cell (0.0052); dendritic cell (0.000901); T cell (0.00002), although both *nod1* and *nlrp3* were expressed in CD_{14}^+ monocytes, CD_{21}^+ B cells, CD_{172}^+ dendritic cells, but not CD_3^+ T cells. *nlrp3* was found to be differentially expressed among PBMC populations, while both *nod1* and *nod2* demonstrated relatively consistent levels of gene expression between cell populations. The relative expression of this gene was found to be most prevalent in cells with phagocytic capacity, such as CD_{14}^+ monocytes, CD_{21}^+ B cells, and CD_{172}^+ dendritic cells, though this gene was also detected in the negative CD3 fraction of cell isolates which contains circulating natural killer

cells and granulocytes. Porcine CD_{14}^{+} monocytes expressed the highest relative expression of *nod1*, *nod2* and *nlrp3* (0.000513; 0.00218; 0.003021, respectively) in comparison to CD_{21}^{+} B cells, and CD_{172}^{+} dendritic cells. Significant levels of *nod1, nod2 and nlrp3* were not detected in peripheral CD_{3}^{+} T cells.

Primer Name	Sequence 5'-3'	Primer Name	Sequence 5'-3'
IL-18 F	GAG GTC TGG CAG TAA CAA TC	NOD1 F	GAG GCA TCC TTG CTG ATC AC
IL-18 R	CAT GTC CAG GAA CAC TTC TC	NOD1 R	CTG TCA TGG TCT GCG ACT TC
NLRP3 F	CCT CTT TGG CCT TGT AAA CC	CD80 F	CTC CCG CCT CTG AAC AAC AC
NLRP3 R	CTC CTG CAT TTC GTG CAA AC	CD80 R	TTG CCC AAG TAT GAG AAC CG
NOD2 F	TGC TCA AGA ATG CCC GCA AG	CD86 F	TGG GTA GCC TTG TGT AGA TG
NOD2 R	CAC CAT CCA CGA GAA GAC AG	CD86 R	CCT ATC CAC CAG ATG AGT TC

Table 2: List of primer sequences for RT-qPCR

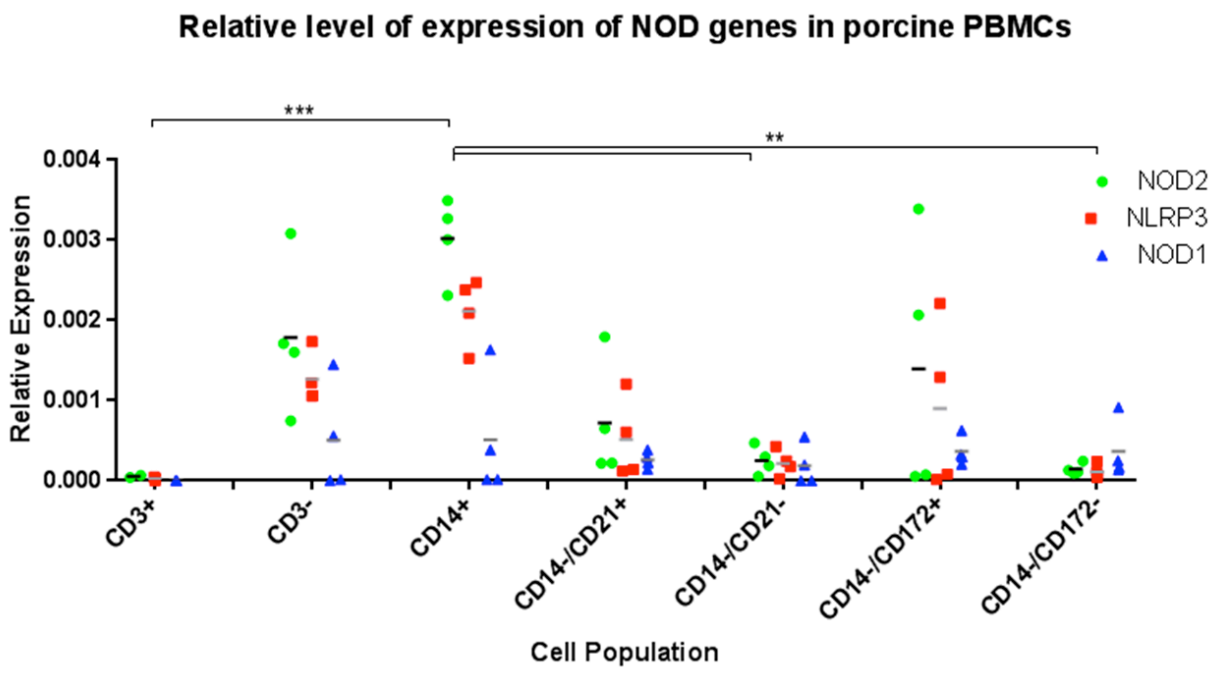


Figure 2: The relative level of expression of *nod1, nod2* and *nlrp3* in porcine PBMCs. Data values are representative of four individual pigs, age six-weeks. Samples were normalized to RPL-19 and relative mRNA expression was calculated using ΔCt values.
Relative to T cells, *nod1* was marginally detected in monocytes, B cells and dendritic cells.

However; statistical analysis did not report a significant difference in the expression of *nod1* mRNA between the various cell populations isolated.

nlrp3 was found to be differentially expressed in the PBMCs populations isolated. The relative expression of this gene was observed to be most prevalent in cells with phagocytic capacity: monocytes, B cells and dendritic cells. CD_{14}^{+} monocytes harbor the greatest level of expression of this receptor (median relative expression = 0.002237). A significant difference exists in the relative expression of *NLRP3* between CD_{14}^{+} monocytes and CD_{3}^{+} T cells ($p < 0.001$, $q = 7.266$). No variance in the expression of *NLRP3* between the antigen presenting cell populations, monocyte, B cell and dendritic cells were identified. The pattern of expression of *NOD2* was

found to be similar to that of *NLRP3*. We observed the presence of *NOD2* in porcine PBMC populations CD_{14}^{+} monocytes, CD_{14}^{-} CD_{21}^{+} B cells, and CD_{14}- CD_{172}+ dendritic cells but not T cells, and found this gene to be most expressed in monocytes relative to the other populations isolated (median relative expression = 0.003140). The prevalence of this receptor was not found to be statistically different among monocytes, B cells or dendritic cells.

5.2 Expression of NOD-like receptors in porcine mucosal and lymphoid tissues

To characterize the expression of NLRs in porcine mucosal and lymphoid tissue, we examined the presence of *nod1, nod2* and *nlrp3* in samples collected from the bronchial mucosa, nasal mucosa, lung, bronchial lymph nodes, mesenteric lymph nodes, Peyer's patches, spleen and gut wall of six-week old pigs. Using RT-qPCR, we measured the relative gene expression of *nod1, nod2* and *nlrp3* and normalized our results to RPL-19 (Figure 3). Our data indicated that tissues associated with the respiratory tract (bronchial mucosa (BM), nasal mucosa (NM), lung (L) and bronchial lymph node (BLN)) expressed the most significant level of the NLR genes and of the three genes examined, *nod1* was found to be most prevalent in these tissues (average expression of *nod1*: BM (0.004379); NM (0.004096); L (0.006187); BLN (0.002248)).

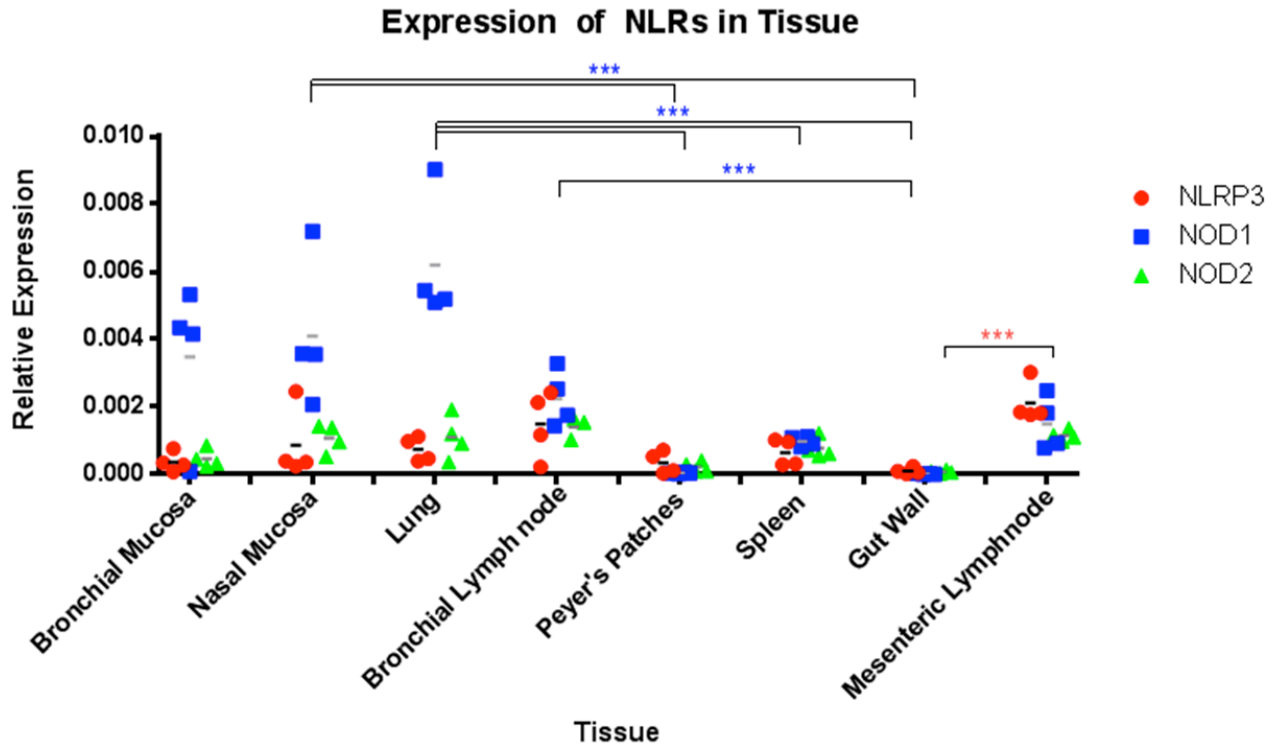


Figure 3: Relative mRNA expression of *nod1, nod2* and *nlrp3* in the mucosal and lymphoid tissue of pigs. Data values are representative of four individual pigs, age six-weeks. Samples were normalized to RPL-19 and mRNA expression was calculated using ΔCt values.

Interestingly, NLR gene expression appeared to be limited in the spleen, Peyer's patches, and gut wall as relative gene expression was only minimally detected in these samples, while the mesenteric lymph node demonstrated levels of *nlr* expression similar to those found in the bronchial lymph node (BLN (*nlrp3* (0.0014869), *nod1* (0.0022489), *nod2* (0.0014047); MLN (nlrp3(0.002112), nod1 (0.0014978), nod2(0.0011528).

nlrp3 expression was found to be significantly different between the bronchial mucosa and mesenteric lymph node ($p < 0.05$, $q = 4.718$), the bronchial lymph node and the gut wall ($p < 0.05$, $q = 5.290$), the mesenteric lymph node and Peyer's patches ($p < 0.05$, $q = 4.861$), and the

gut wall and mesenteric lymph node ($p < 0.01$, $q = 6.863$). No significant variation in the expression of NLRP3 was observed when comparing mucosal and lymphoid tissues of the respiratory tract. *NOD1* expression was observed to be the highest relative to *nod2* and *nlrp3*, and most prevalent in the respiratory tract mucosa (median mRNA expression: bronchial mucosa = 0.004251, nasal mucosa =0.003561, lung = 0.005319). Comparison of gene expression between various tissues found that areas of the respiratory and gastrointestinal tracts differed from each other in respect to the prevalence of this receptor. Significant differences were observed between the bronchial mucosa and Peyer's patches ($p <0.001$, $q = 7.464$), the bronchial mucosa and gut wall ($p< 0.001$, $q = 8.842$), the nasal mucosa and Peyer's patches ($p < 0.001$, $q = 8.383$), the nasal mucosa and spleen ($p < 0.05$,$q = 5.168$), the gut wall and nasal mucosa ($p < 0.001$, $q = 9.761$), the bronchial lymph node and lung ($p < 0.05$, $q = 4.708$), the lung and Peyer's patches ($p < 0.001$, $q = 10.68$), the lung and spleen ($p < 0.001$, $q = 7.464$), the lung and gut wall ($p < 0.001$, $q = 12.06$), the lung and mesenteric lymph node ($p < 0.01$, $q = 6.431$), the bronchial lymph node and Peyer's patches ($p < 0.01$, $q = 5.971$), the bronchial lymph node and gut wall ($p < 0.001$, $q = 7.349$), and the gut wall and mesenteric lymph node ($p < 0.05$, $q = 5.627$). The expression of *nod2* was similar to *nlrp3* among the various tissues. Differential expression of *nod2* was found between the bronchial mucosa and the bronchial lymph node. ($p < 0.05$, $q = 5.434$), the nasal mucosa and Peyer's patch ($p < 0.05$, $q = 5.616$), the nasal mucosa and gut wall ($p < 0.001$, $q = 7.139$), the lung and Peyer's patch ($p < 0.05$, $q = 5.235$), the lung and gut wall ($p < 0.01$, $q = 6.758$), the bronchial lymph node and Peyer's patch ($p < 0.01$, $q = 6.932$), the bronchial lymph node and gut wall ($p < 0.001$,$q = 8.342$), the Peyer's patch and the mesenteric lymph node ($p < 0.01$, $q = 5.997$), the spleen and the gut wall ($p < 0.05$, $q = 5.521$), and the mesenteric lymph node and gut wall ($p < 0.001$, $q = 7.519$).

5.3 IL-1β production by cultured monocytes

Inflammasome activity and IL-1β secretion are thought to be tightly regulated by multiple pro-inflammatory signaling pathways, which include cross-talk with TLRs. In order to determine whether polyphosphazenes PCEP or PCPP could induce the secretion of IL-1β, ELISA was performed at 12 and 24 hours on cultured monocytes (**Figure 3, 4**). Resting monocytes cultured in RPMI complete media were used as control. Cells cultured alone with either muramyl di-peptide (MDP; Sigma-Aldrich), PCEP, PCPP, or a combination of CpG-ODN and poly I:C (TLR) represent the one signal model of activation. Monocytes co-cultured with TLR and either PCEP, PCPP or MDP represent the two signal model of activation. The concentration of IL-1β was increased in the supernatant of monocytes cultured for 24 hours as compared to 12 hours. Additionally, it was observed that monocytes cultured with one signal did not significantly secrete IL-1β relative to our control or to other groups. However, monocytes co-cultured with TLR and either polyphosphazene or MDP resulted in median cytokine concentrations of 763.7 pg/mL (TLR-PCEP), 553.6 pg/mL (TLR-PCPP) and 231.8 pg/mL (TLR-MDP) and significantly enhanced the secretion of IL-1β relative to control: TLR-PCEP ($p < 0.001$, $q = 8.619$), TLR-PCPP ($p < 0.001$, $q = 7.304$) and TLR-MDP ($p < 0.05$, $q = 5.099$). We found that cells cultured with TLR-PCEP appear to produce the greatest titers of IL-1β, relative to TLR-PCPP or TLR-MDP, but no statistical differences were reported between these groups.

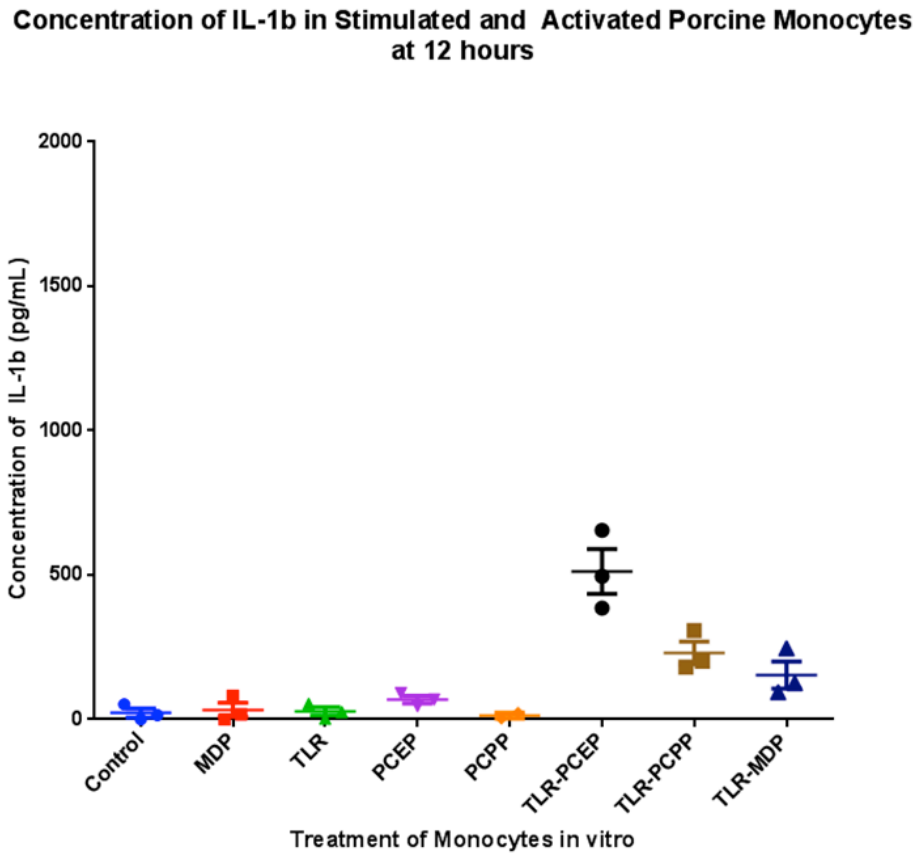


Figure 4: Production of IL-1β for 3 pigs at 12 hours. Porcine monocytes were plated at a concentration of $1x10^6$ cells per mL at a volume of 500uL in 48 well plates. Monocytes were cultured with MDP (1ug/mL), CpG (2.5ug/mL) + Poly I:C (50ug/mL), PCEP(25ug/mL), PCPP(25ug/mL). Expression of IL-1β was measured at 12 hrs.

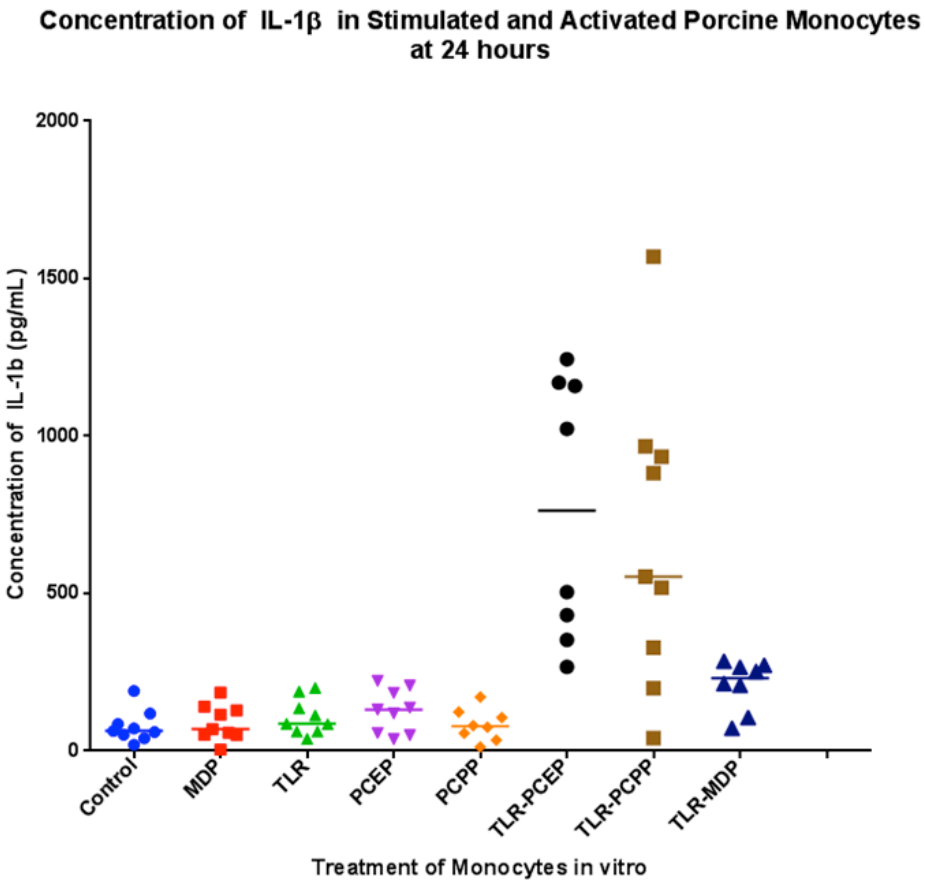


Figure 5: Production of IL-1β . Porcine monocytes were plated at a concentration of $1x10^6$ cells per mL at a volume of 500uL in 48 well plates. Monocytes were cultured with MDP (1ug/mL), CpG (2.5ug/mL) + Poly I:C (50ug/mL), PCEP(25ug/mL), PCPP(25ug/mL). Expression of IL-1β was measured at 24hrs (8).

5.4 IL-18 cytokine production by cultured monocytes

Optimization of an IL-18 ELISA was completed using a checkerboard titration method. ELISA was performed on the supernatant of monocytes in order to determine whether IL-18 was produced from cells co-cultured with polyphosphazene. Although both diluted and undiluted samples of supernatant were used in this study. In reflection of the standard curve, which was generated from these assays, it was found that IL-18 concentrations fell below the range required to produce a signal for this cytokine. Of the 12 pigs that were analyzed, only 4 pigs demonstrated the production of IL-18 at detectable concentrations of roughly 100-250 pg/mL.

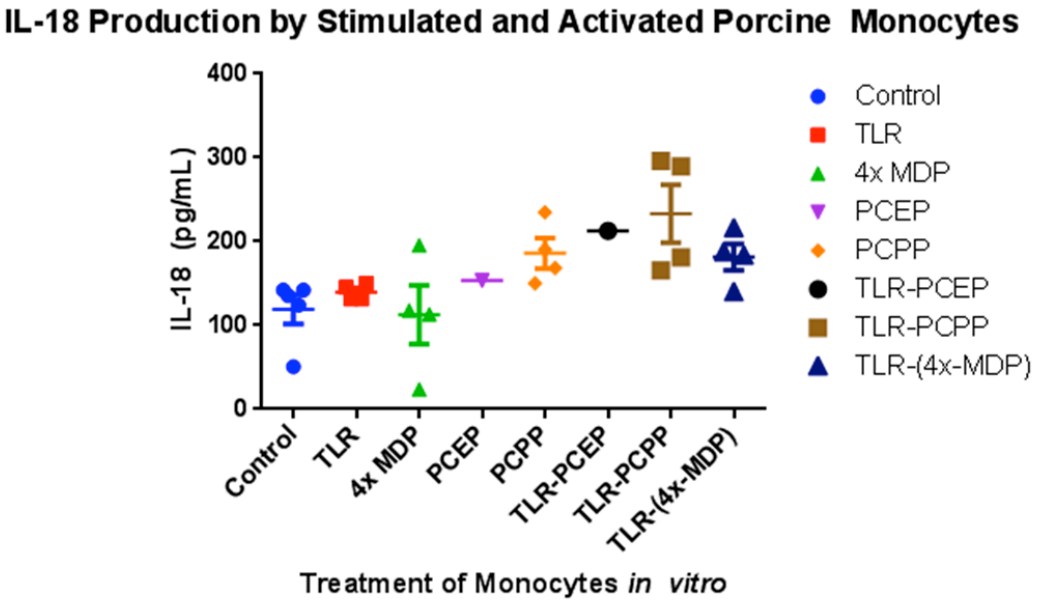


Figure 6: IL-18 production by stimulated and activated porcine monocytes in response to 4x concentration MDP.

5.5 Production of IL-6, IL-12 and IL-10

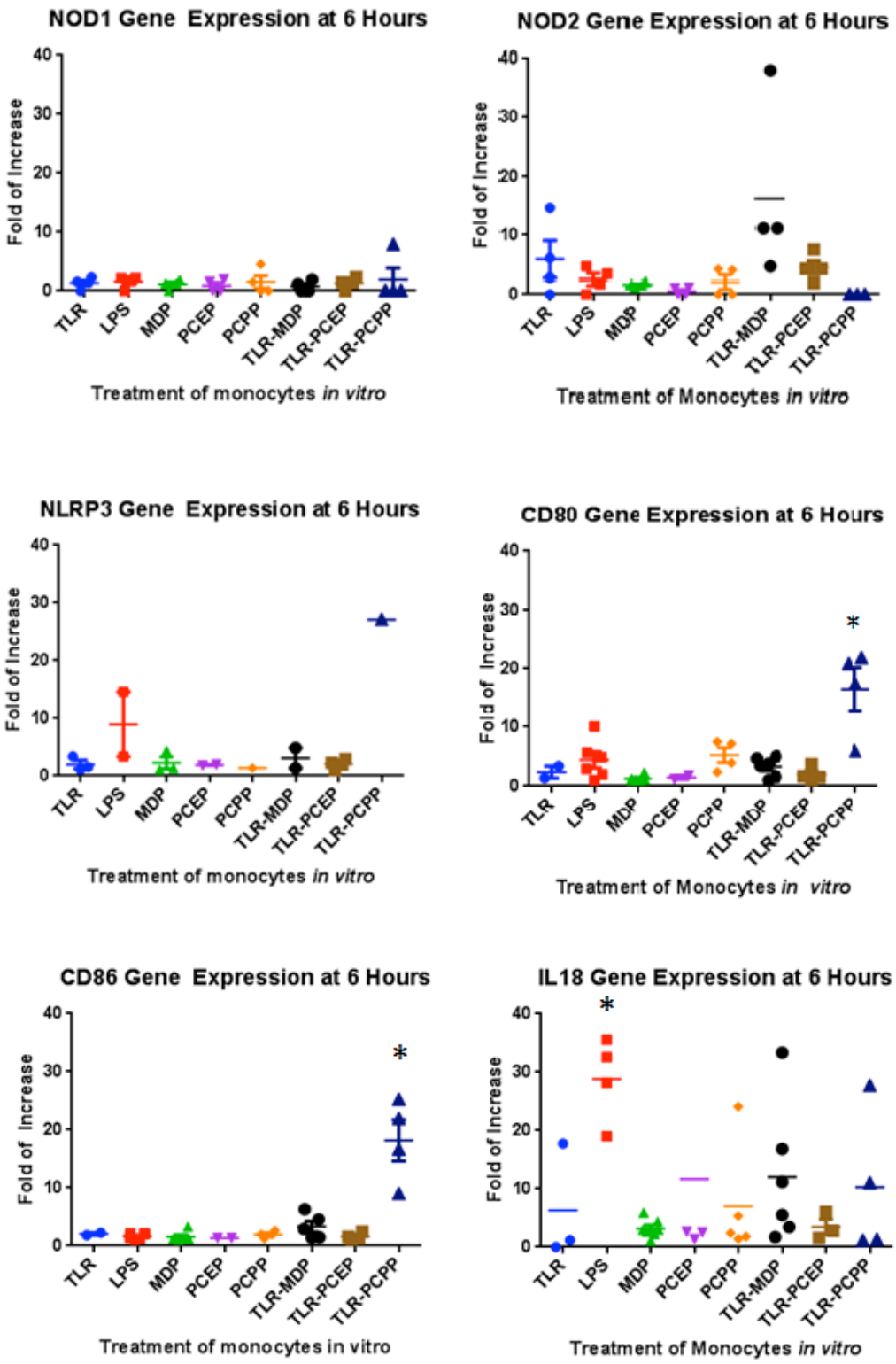
To determine whether other inflammatory cytokines were being secreted by monocytes cultured with polyphosphazene, ELISA for IL-6, IL-12 and IL-10 were performed on the supernatant of 8 pig experiments. Similarly to IL-18, the concentration of these cytokines fell below the standard curves generated in each experiment, and we therefore did not observe the significant production of either IL-6, IL-12 or IL-10 by these cells.

5.6 RT-qPCR in stimulated and activated porcine monocytes

RT-qPCR was performed at 6 hours post stimulation of monocytes cultured with 1 or 2 signals to determine whether inflammasome related genes or cell activation markers were up-regulated in the presence of polyphosphazene**.** We found that stimulation with lipopolysaccharide (LPS)(Sigma-Aldrich) or TLR ligands (CpG-ODN, poly I:C) induced the expression of *IL-18* mRNA in porcine monocytes relative to cells cultured with MDP, PCEP or PCPP alone. Furthermore, we observed that cells co-cultured with TLR, and either MDP, PCEP or PCPP induce the expression of IL-18 relative to cells cultured with only MDP, PCEP or PCPP. The expression of cell maturation molecules CD80 and CD86 did not demonstrate any significant differences between any of the treatment groups, however cells co-stimulated with TLR and MPDP appeared to enhance the expression of CD86 in porcine monocytes relative to MDP alone ($p<0.01$, $q=5.433$). CD86 did not appear to be up-regulated in response to any other treatment. The expression of inflammasome related genes *nod1*, *nod2* and *nlrp3* appeared to be limited in our monocytes as CT values obtained from RT-qPCR were consistently demonstrated to be

above 35, indicating relatively little presence of the genes. In values obtained from 4 pigs, we found *nod2* expression to be up-regulated in monocytes co-cultured with TLR ligands and MDP however, no statistically significance could be found between any of our treatments.

The expression of inflammasome related genes *nod1*, *nod2* and *nlrp3* appeared to be limited in our monocytes, as CT values obtained from RT-qPCR were consistently demonstrated to be above 35 (Figures 9,10,11). In values obtained from 4 pigs, we found that *nod2* expression was upregulated in monocytes co-cultured with TLR ligands and MDP (Figure 10), however no statistical significance was reported between any of our treatments.



Figures 7-12: RT-qPCR results using porcine primers specific for IL-18, CD86, CD80, NOD1, NOD2 and NLRP3. Results are normalized to RPL-19

5.7 Caspase inhibitor assay

A soluble inhibitor for caspase-1 (CI)(Caspase-1 Inhibitor VI, Calbiochem, EMD Biosciences, La Jolla, CA) was used to determine if IL-1β cytokine secretion could be inhibited by cells co-cultured with TLR and polyphosphazene. CI was solubilized in dimethyl sulfoxide (DMSO)(Sigma-Aldrich) and concentrations of 25uM, 50uM, 75uM and 100uM were evaluated. CI was added to the wells at 2 hours prior to stimulation with TLR ligands and ELISA for IL-1β was used to detect the presence of protein in the supernatant of monocytes. We found that cytokine production was not significantly inhibited in monocytes cultured with two signals (TLR-MDP, TLR-PCEP) and 25uM, 50uM, and 75uM CI**.** Relative to our control, IL-1β concentration was reduced in cells cultured with TLR-PCEP and 100uM CI.

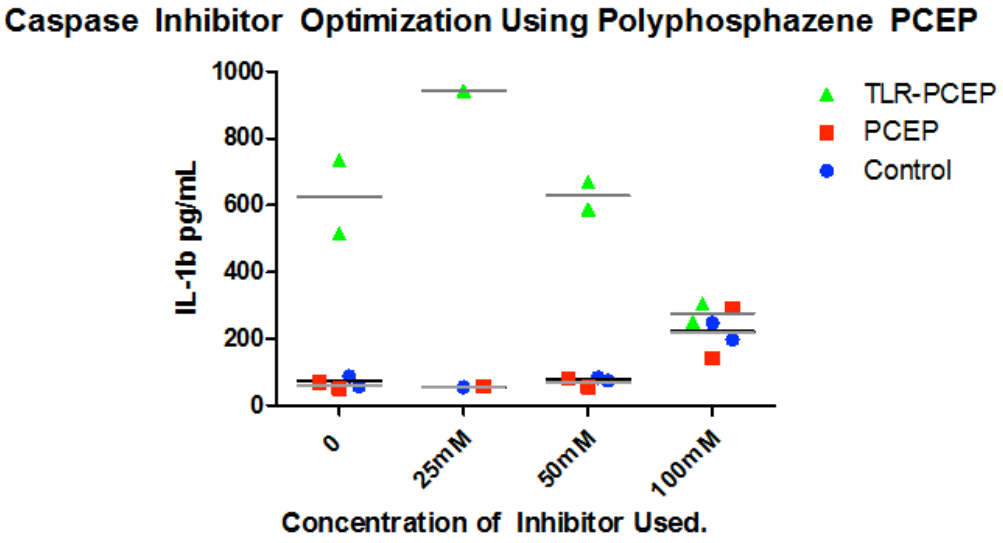


Figure 13: Caspase Inhibitor Optimization assay of monocytes cultured at a concentration of $1x10^6$ cells/mL in a volume of 200uL in a 48well plate with 50uM, 75um and 100um of inhibitor at 2 hours prior to stimulation with CpG (concentration) and Poly I:C (concentration) and PCEP with IL-1β ELISA.

6.0 DISCUSSION AND CONCLUSIONS

According to the World Health Organization (WHO 2014), vaccination is the most effective method of preventing infectious disease. In recent years, vaccination campaigns have significantly contributed to the elimination and eradication of several deadly human and veterinary diseases including pertussis, polio, measles, mumps, rubella, small pox, and rinderpest. Recently, there has been an emerging interest in the porcine immune system due to the economic importance of livestock industry and the knowledge that pigs share similar genetics, anatomical and physiological structure to humans (Tohno et al., 2011). Pigs have provided an excellent model for the investigation of several human pathogens including *B. pertussis*, influenza, respiratory syncytial virus and rotavirus (Meurens et al., 2012), and due to a greater than 80% shared similarity of analyzed immune parameters (Dawson et al., 2011), pigs are a more accurate predictor of therapeutic outcome in humans than most other animal models (Meurens et al., 2012).

The NLRs are a recently identified family of cytosolic PRR receptors that are thought to play a critical role in the innate immune response (Schroder and Tschopp, 2010; Correa et al., 2012). NOD1, NOD2 and NLRP3 are the most characterized of the NLRs (Martinon et al., 2009). Antigenic stimulation of NOD1, NOD2, and NLRP3 as a result of infection, tissue or cell damage can induce the oligomerization of NLRs in to an 'inflammasome' complex within the cell which regulates the catalytic cleavage and release of IL-1β, IL-18 and IL-33 by caspase-1. The recent identification of nucleotide sequences specific for porcine NLRs have provided a means to investigate inflammasome gene expression in pigs using methods such as RT-qPCR

(Ensembl; NCBI). However despite the availability of these sequences, the mechanisms of activity of the inflammasomes are not well characterized in pigs. For this reason, we chose to evaluate the relative gene expression of NOD1, NOD2 and NLRP3 in porcine immune cell populations and mucosal and lymphoid tissue. We rationalized that these sequences would allow us to detect whether these receptors are present in the pig, and if they could be induced in the presence of a novel vaccine adjuvant, polyphosphazene.

In a preliminary investigation using RT-qPCR to determine the relative level of gene expression of *nod1*, *nod2* and *nlrp3* in porcine immune cells, we found that gene expression of these receptors were limited to monocytes, B cells and dendritic cells.

We also found that the expression of *nod1*, *nod2* and *nlrp3* was greatest in CD_{14}^{+} porcine monocytes as compared to CD_{172}^{+} dendritic cells and CD_{21}^{+} B cells. Although it is unclear why the relative prevalence of these receptors were found to be the most significant in monocytes, it may be possible that the elevated existence of NOD1, NOD2 and NLRP3 in monocytes could provide a specific mechanism of surveillance to various stimuli throughout the body. Furthermore, circulating CD_{14}^{+} monocytes which are actively recruited to sites of inflammation, and are capable of differentiating in to both tissue resident macrophages and dendritic cells may represent a novel target for innovative vaccine strategies (Ezquerra et al., 2009).

In order to determine whether *nod1*, *nod2* and *nlrp3* could be detected in porcine mucosal and lymphoid associated tissue, we used RT-qPCR to evaluate the relative gene expression of these NLRs in tissue samples obtained from the bronchial mucosa, nasal mucosa, lung, bronchial lymph nodes, Peyer's patches, spleen, gut wall and mesenteric lymphnodes of 4 adult pigs. We found that *nod1* gene expression, relative to *nod2* and *nlrp3*, was most prevalent in the

respiratory associated tissues of pigs (bronchial mucosa, nasal mucosa and lung). These areas represent a major site of entry for infectious pathogens, and the presence of PRRs in both phagocytic epithelial cells and antigen presenting cells offer a valuable first line of defense against infection. Previous studies have shown that NOD1 and NOD2 are important regulators of pulmonary innate immunity and pathogen clearance of *Legionella pneumophila* and *Chlamydia pneumophila* respiratory infections (Shimada et al., 2009; Berrington et al., 2010) and it is known that NOD1 recognizes conserved structures belonging to the cell wall of Gram negative bacteria (Chamaillard et al., 2003). We cannot conclude why the relative expression of *nod1* was highest in the lungs, nasal mucosa and bronchial mucosa of our pigs, though it is possible that previous or repetitive exposure to various antigenic stimuli during growth and development may have resulted in an overall increase in *nod1* expression at these sites. It is also possible that the specific expression of *nod1* in the respiratory tissues provides a strategic mechanism to rapidly detect and eliminate harmful Gram negative bacteria at the first signs of infection.

Interestingly, we found that NLR expression was limited in the spleen and Peyer's patches of adult pigs which was surprising as these areas are notoriously dense in monocytes, macrophages and dendritic cells. It is known that NLR expression can be induced following TLR stimulation (Martinon et al., 2009), and that age related differences exist in the expression of NLRP3 in the spleen, Peyer's patches and mesenteric lymphnodes (Tohno et al., 2011). Studies have also indicated that *nod1*, *nod2* and *nlrp3* expression is highly restricted among various types of tissue (McDaniel et al., 2008). It is possible that cells located at these sites may not have received sufficient TLR-priming to induce a detectable NLR expression, or that *nod1*, *nod2* and *nlrp3* expression may be minimized at these sites as a means to limit unnecessary inflammation under

steady state conditions, as it is suspected the NLRs are involved in the pathology of several autoimmune diseases, chronic inflammation syndromes and cancer (Schroder and Tschopp, 2010).

Polyphosphazenes are a relatively new class of synthetic polymer adjuvant. In previous studies, polyphosphazenes have shown to promote the innate immune response to a variety of bacterial and viral antigens (Eng et al., 2010). Currently the mechanisms by which polyphosphazenes exert their adjuvant effect are not well understood, but are not thought to occur as the result of a depot formation at the site of injection (Andrianov, 2006). Based on our RT-qPCR results which determined that NLRs were most prevalent in monocytes, we chose to investigate whether polyphosphazene PCEP or PCPP could induce the expression of *nod1, nod2* and *nlrp3* genes in porcine monocytes. Furthermore, we wanted to identify whether stimulation of monocytes in the presence of both a TLR-agonist and polyphosphazene could induce the secretion of pro-inflammatory cytokines IL-1β and IL-18. We found the monocytes stimulated with polyphosphazene alone did not significant induce the upregulation of *nod1*, *nod2* or *nlrp3*, however when combine with a second signal (CpG-ODN and poly I:C), *nod2* gene expression could be induced to expression levels similar to treatment with the positive control TLR-MDP. Because gene expression does not always correlate protein expression, we examined the secretion of inflammasome related cytokines IL-1β and IL-18 in the supernatant of cultured monocytes. We observed a significant secretion of IL-1β, but not IL-18, in response to a two-signal model of stimulation of porcine monocytes with polyphosphazene. We also observed the relative expression of IL-18 mRNA to be induced by the stimulation of monocytes with TLR-PCPP and TLR-PCEP, but not in response to polyphosphazene or TLR agonists alone. Our

results suggest that either polyphosphazene was not present within the monocytes, or that stimulation with TLR-PCEP and TLR-PCPP was not sufficient to induce the formation of the inflammasome complex and result in the catalytic cleavage of IL-18 in to its bioactive form. It is also possible that polyphosphazenes do not act as an agonist for NOD1, NOD2 or NLRP3, and may utilize an intracellular pathway other than the NLRs to exert their adjuvant effect.

In our final experiments, we looked to determine whether IL-1β secretion could be inhibited in monocytes that were co-cultured with TLR-PCEP and a soluble caspase inhibitor (CI). We found that IL-1β secretion was not significantly different between cells that were co-cultured with MDP, our positive control TLR-MDP, and either 50uM, 75u or 100uM CI. Though, we did observe IL-1β secretion to be reduced in monocytes which were co-cultured with TLR-PCEP and 100uM CI. After viewing these cells with light microscopy, we suspected that this reduction in IL-1β was due to a toxic level of DMSO which was killing the cells, rather than inhibiting caspase itself. It is possible that NLR specific IL-1β and IL-18 secretion in monocytes may be dependent on the presence of additional external factors, such as cell-cell contact or various metabolic products like ATP (O'Hagan et al., 2012; Piccini et al., 2008). It is also possible that our monocytes were not cultured for a sufficient length of time to induce the synthesis of IL-18. Furthermore, the maturity status of the monocytes may also have played a role in the response to TLR-polyphosphazene stimulation in this study, as PCEP induced IL-1β and IL-18 secretion have been reported in murine dendritic cells (Awate et al., 2012; Awate 2012).

In summary, it is thought that combination adjuvants exert their effect through stimulation of multiple PRR pathways. The novel adjuvant platform developed at VIDO-InterVac, which

consists of CpG-ODN, poly I:C, IDRs and polyphosphazene has shown a great potential as a safe and effective adjuvant to a variety of viral and bacterial antigens in many animal studies (Dar et al., 2012; Eng et al., 2009, Eng et al., 2010; Garlapati et al., 2011; Kovacs-Nolan et al., 2009; Gracia et al., 2011). Although the mechanism of action of polyphosphazene is not currently understood, we found that a two-signal model of cell stimulation with polyphosphazene had the potential to induce the secretion of IL-1β but not IL-18 by porcine monocytes. These results suggest that a signaling cascade which acts independently of the NLRs may mediate the adjuvant activity of polyphosphazene, or that the production of IL-1β and the formation of a positive feedback loop which involves the recruitment of cells and cell-to-cell interaction at the site of immunization may play an important role in the mechanism of action of polyphosphazene-based adjuvants. Further studies which evaluate the immunological effects of PCEP and PCPP in various cell types in pigs may provide a greater insight in to the precise mechanism of action of polyphosphazenes and in turn will undoubtedly aide in the development and safety of many novel vaccine formulations.

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